1 Vectors for the transformation of plant cells using *Agrobacterium*

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1.1 GENERAL INTRODUCTION

1.1.1 Tumour induction by *Agrobacterium*

Agrobacterium tumefaciens and *A. rhizogenes* are soil bacteria which induce crown gall and hairy root disease respectively at wound sites on dicotyledonous plants. Only a very few monocotyledonous plants in the families *Liliaceae* and *Amaryllidaceae* have been reported to be weakly susceptible to crown gall induction (see reviews below). The reason for this limit in host range is not currently understood. Once initiated, tumorous growth can continue in the absence of the bacterium and tumour tissue can grow axenically in tissue culture in media lacking exogenous supplies of auxins and cytokinins, which are normally required to promote growth of plant tissues *in vitro*. Tumour tissues synthesize novel amino acid and sugar derivatives known collectively as opines. The type of opine synthesized in the tumour (for example, nopaline, octopine, agrocinopine, mannopine and agropine) is dependent on the strain of *Agrobacterium* that initiated tumour formation. Octopine and nopaline are two types of opines derived from arginine and among the easiest to detect in crown gall tissue. Consequently, many common *Agrobacterium tumefaciens* strains are designated as octopine or nopaline types. Agropine, a sugar derivative, is commonly found in hairy root tumours induced by *A. rhizogenes*. The *Agrobacterium* responsible for tumour formation selectively catabolizes the opine whose synthesis it has induced, using it as a source of carbon and nitrogen.

Both tumour induction and opine synthesis are associated with the presence within the bacteria of a megaplasmid, the Ti (*Tumour inducing*) plasmid in the case of *A. tumefaciens* and the Ri (*Root inducing*) plasmid in *A. rhizogenes*. The molecular biology of tumour induction is reviewed in the following: Kahl and Schell (1982), Caplan *et al.* (1983), Gheysen *et al.* (1985) and Stachel and Zambryski (1986).

1.1.2 Ti plasmids of *Agrobacterium tumefaciens*

Ti plasmids (Fig. 1.1A), found in all virulent strains of *A. tumefaciens*, are around 200–250 kilobases (kb) in size and are stably maintained in the *Agrobacterium* at temperatures below 30 °C. Ti plasmids found in different strains of *Agrobacterium* have four regions of homology, as judged by DNA–DNA hybridization and heteroduplex mapping. Genetic analysis has shown that two regions, the T- (*Transferred*) DNA and the *vir* (*virulence*) region are associated with tumour formation, whereas the other two are involved with conjugative transfer and the replicative maintenance of the plasmid within the *Agrobacterium*.

During tumour formation a defined sequence of Ti plasmid, the T-DNA, is transferred to the plant cell and integrated into the plant nuclear genome. The T-DNA is stable within the plant genome and hybridization of a Ti plasmid-specific probe to tumour DNA has shown that the T-DNA found in the plant cell is

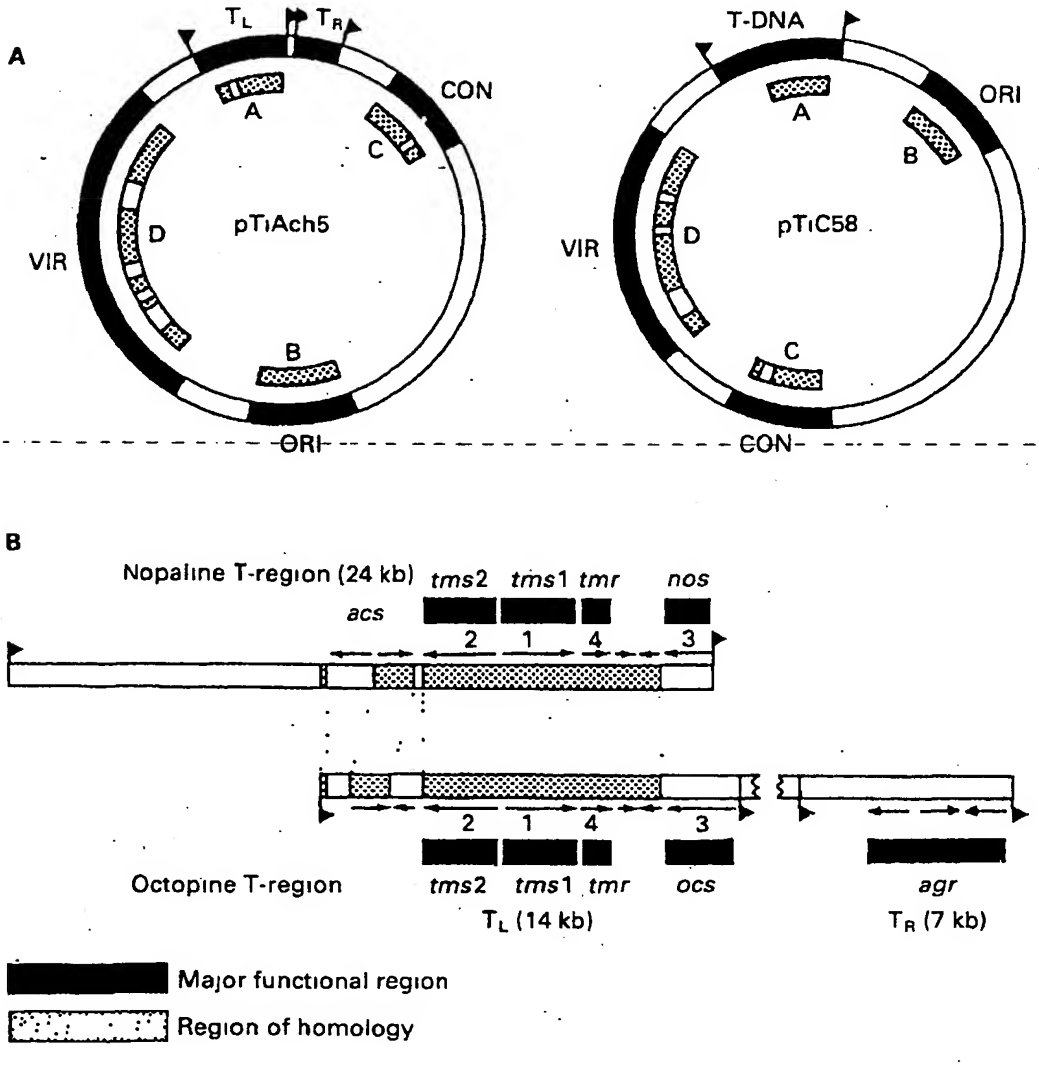
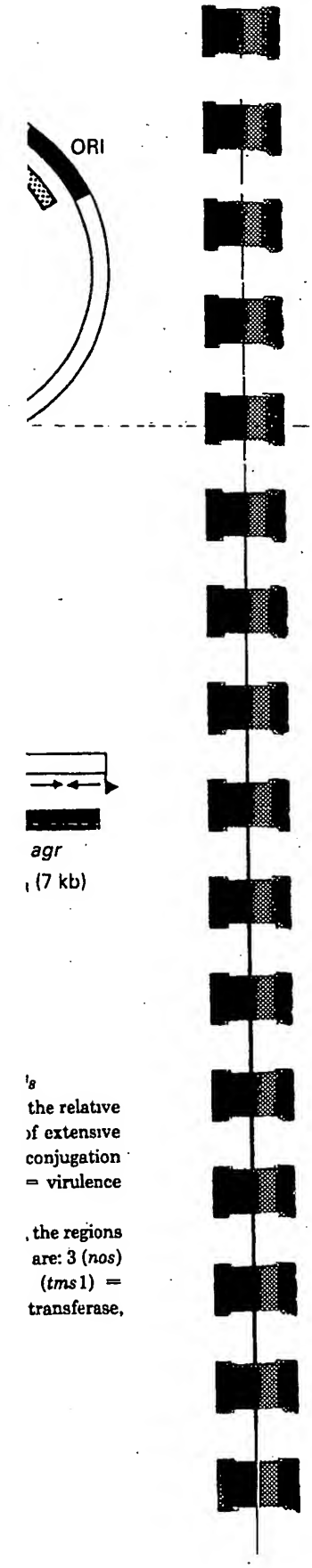


Fig. 1.1 *General organization and expression of octopine- and nopaline-type Ti plasmids*
A Map of an octopine-type (pTiAch5) and a nopaline-type (pTiC58) Ti plasmid showing the relative positions and sizes of the major functional regions. Shaded areas indicate the regions of extensive homology between the two plasmids ‖ = 25 bp direct repeats; CON = regions encoding conjugation functions, ORI = region encoding replication functions and origin of replication, VIR = virulence region; T-DNA, T_L, T_R = regions containing T (Transferred)-DNA.
B Maps of a nopaline- and octopine-type T region showing the major functional domains, the regions of homology and transcripts. Products of transcription which have been identified to date are. 3 (*nos*) = nopaline synthase; 3 (*ocs*) = octopine synthase; *acs* = agrocinopine synthase; 1 (*tms* 1) = tryptophan mono-oxygenase; 2 (*tms* 2) = indole-3-acetamide hydrolase; 4 (*tmr*) = DMA transferase; *agr* = 3 transcripts required for agropine synthesis.



co-linear with the T-DNA found in the Ti plasmid of the *Agrobacterium*, indicating that no major rearrangements of the sequence take place during establishment of the tumour. One or more copies of the T-DNA can be present in the plant DNA and, although multiple T-DNA copies can occur in tandem repeats, they can also be separate and linked to different regions of plant DNA. The site of integration of T-DNA into plant DNA is apparently random. Regions homologous to the T-DNA are found on different Ti plasmids (Fig. 1.1B). In commonly used nopaline strains of *A. tumefaciens* the T-DNA region is around 24 kb. In some octopine-type crown galls two non-contiguous segments are found, the T_L and T_R. T_L (14 kb) is present in all transformed cell lines and is functionally equivalent to the T-DNA found in nopaline cell lines. T_R (7 kb), which originates from the right of the T_L-DNA on the Ti plasmid, is not found in all tumour lines but when it is its copy number can differ from that of T_L, suggesting an independent transfer process.

Within the tumour cells the T-DNA is transcribed (Fig. 1.1B) to produce a variety of polyadenylated mRNAs. The levels of the T-DNA transcripts which accumulate are relatively low compared with other plant mRNAs and the relative abundance of each can differ. Sequencing of the nopaline-type T-DNA has revealed 13 large open-reading frames, whilst there are eight and six large open-reading frames in octopine-type T_L- and T_R-DNA respectively. The transcripts on the right-hand side of the nopaline T-DNA are functionally equivalent to those on T_L-DNA (Fig. 1.1B). The overall organization of the T-DNA genes and their flanking regions are similar to those found in eukaryotic genomes, although they do not contain introns. Sequence comparisons, deletion and transposon mutagenesis as well as overproduction of the individual gene products in *E coli* have been used to identify the functions of several of the gene products encoded by T-DNA. One gene in the octopine T_L region (transcript 3) encodes octopine synthase. In nopaline Ti plasmids, opine synthase genes include nopaline synthase (*nos*) and agrocinopine synthase (*acs*). In octopine Ti plasmids the T_R region encodes two proteins responsible for the synthesis of mannopine and one gene product responsible for the conversion of mannopine to agropine. The *tmr* locus (transcript 4) encodes an enzyme involved in the synthesis of cytokinin, and mutations here result in root proliferation from crown galls induced on some species (rooty mutants). The *tms*1 and *tms*2 loci (transcripts 1 and 2) are involved with the unregulated synthesis of auxins, and mutations in either of these result in shoot proliferation from crown galls on many types of plants (shooty mutants). Thus, the T-DNA contains genes (*tms*1, *tms*2 and *tmr*) whose products override the normal regulation of plant metabolic pathways involved in the synthesis of phytohormones and this results in the oncogenic phenotype. Hence, these genes (*tms*1, *tms*2 and *tmr*) can be considered to be *oncogenes* (see 2.1 for more detail). However, it must be noted that the genes encoded by the T-DNA are not required for the transfer of the T-DNA to the plant cell, nor its stable maintenance in the plant genome.

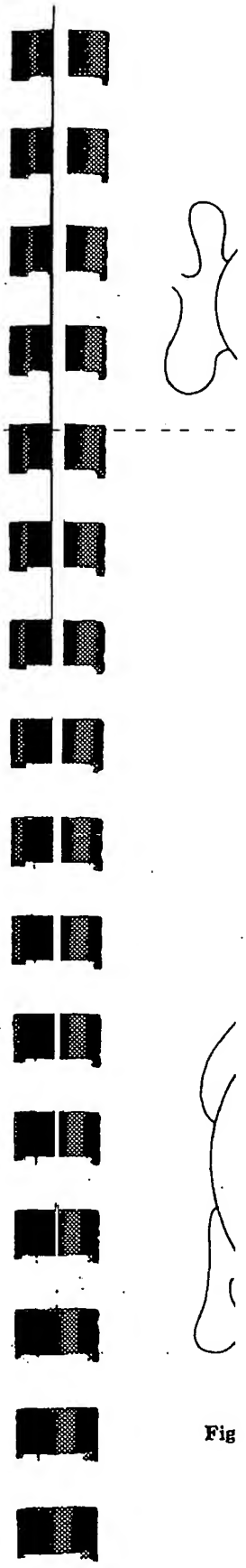
1.1.3 Ri plasmids of *Agrobacterium rhizogenes*

Initiation of hairy root disease by *A. rhizogenes* is analogous to transformation by *A. tumefaciens*. Under the control of a virulence region, two separate T-DNA regions of the Ri plasmid are transferred to the plant genome (Huffman *et al.*, 1984; De Paolis *et al.*, 1985). These are termed T_L (T-left T-DNA) and T_R (T-right T-DNA). The T_R T-DNA contains genes for opine production (mannopine or agropine) and strains are characterized by their particular opine genes (De Paolis *et al.*, 1985). In addition the T_R T-DNA contains two genes which code for auxin synthesis. These genes are highly homologous to the auxin genes of *A. tumefaciens* and have been shown to be capable of complementing *A. tumefaciens* strains carrying mutations in their auxin genes (Offringa *et al.*, 1986). The T_L T-DNA, on the other hand, does not have homology with the T-DNA of *A tumefaciens* (Huffman *et al.*, 1984). The entire T_L T-DNA of *A. rhizogenes* has been sequenced and contains at least 11 open-reading frames, similar to those found in eukaryotic genomes, which have the necessary promoter and polyadenylation elements needed to function upon transfer into the plant genome (Simpson *et al.*, 1986). These genes are not homologous to any known genes; nor are there significant homologies with (octopine type) T-DNA from *A. tumefaciens*. T_R T-DNA is not absolutely required for the maintenance of the hairy root phenotype. However, strains of *Agrobacterium* possessing both T_L and T_R T-DNA are more virulent, on a wider range of plant species, than are strains possessing only one T-DNA (Vilaine and Casse-Delbart, 1987).

1.1.4 Mechanism of T-DNA transfer

T-DNA regions in both *A. tumefaciens* and *A. rhizogenes* are flanked by 25 base pair (bp) direct repeats (Fig. 1.1) and the endpoints of integrated T-DNA in the plant genome are found close to these sequences. The consensus sequence of the T-DNA border is GGCAGGATATT$^{C/G\ A/G}$ G$^{T/G}$ TCTAA$^{A/T\ T/C}$. Most studies of the mechanism by which T-DNA is transferred from the *Agrobacterium* to the plant cell have been carried out with *A. tumefaciens*. The removal of the right border of a nopaline-type Ti plasmid abolishes tumour formation; but when a 25 bp oligonucleotide homologous to the right border is cloned in the correct orientation into a Ti plasmid lacking the right border, tumour formation is restored (Wang *et al*, 1984), suggesting that the 25 bp repeat sequences are both polar and *cis*-acting.

Contact of the *Agrobacterium* with compounds released from wounded plant tissue results in the transcription of the *vir* region of the Ti plasmid (Fig. 1.2). One specific chemical highly active in this respect has been identified as acetosyringone (Stachel *et al.*, 1986). *Vir*A and *vir*G are expressed in vegetatively growing bacteria, although *vir*G is only transcribed at a low level. When *Agrobacterium* is exposed to wounded plant cell exudates, or pure acetosyringone, the *vir*A gene product (possibly membrane-associated) is thought to recognize and interact with acetosyringone and transmit this extracellular signal intracellularly, resulting in



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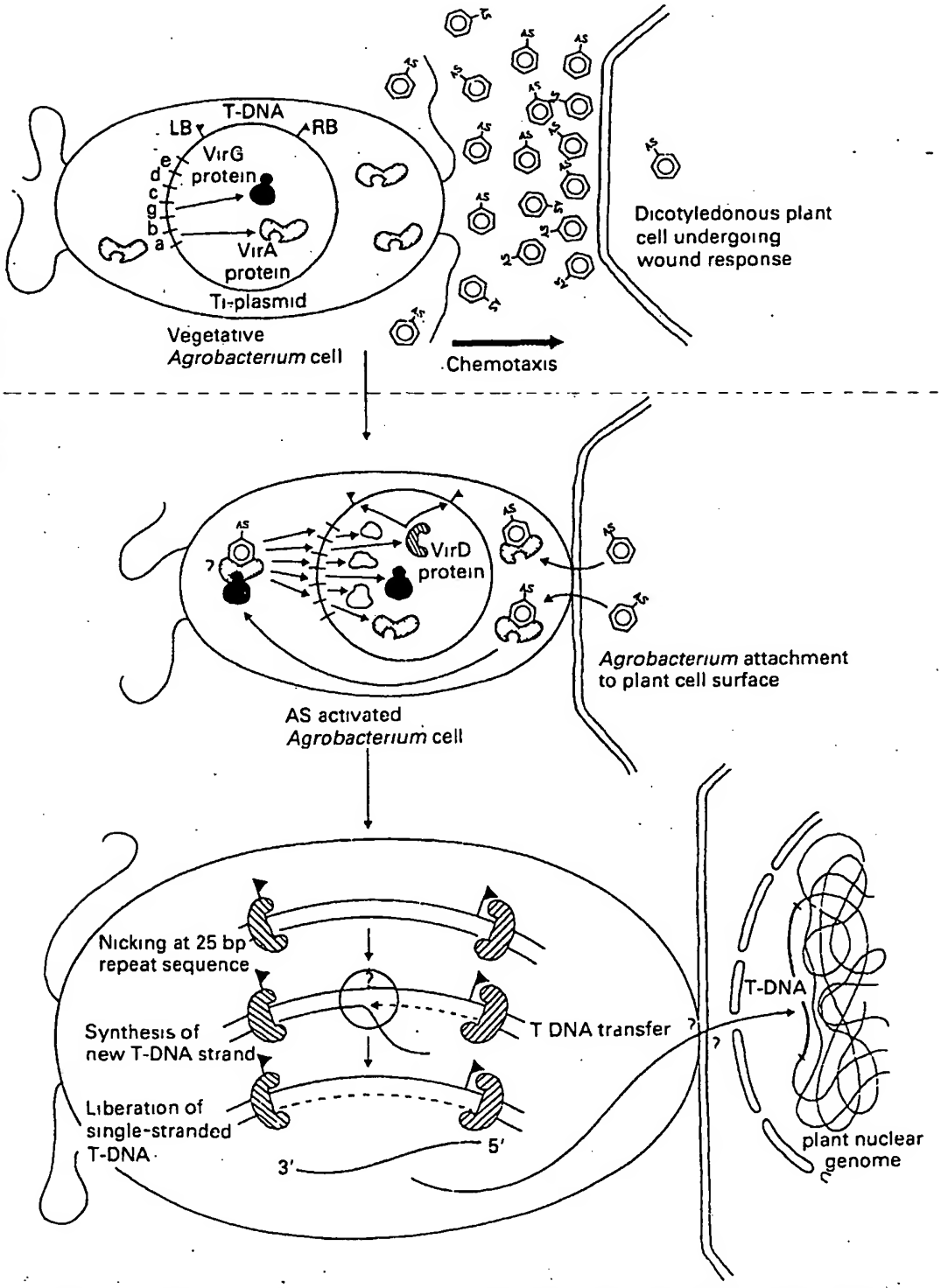


Fig. 1.2 *Agrobacterium/plant interaction and mechanism of T-DNA transfer (AS = acetosyringone)*

the activation of the *vir*G gene product. The altered *vir*G protein then activates the rest of the virulence genes (*vir*B, C, D and E), as well as elevating transcription from the *vir*G locus.

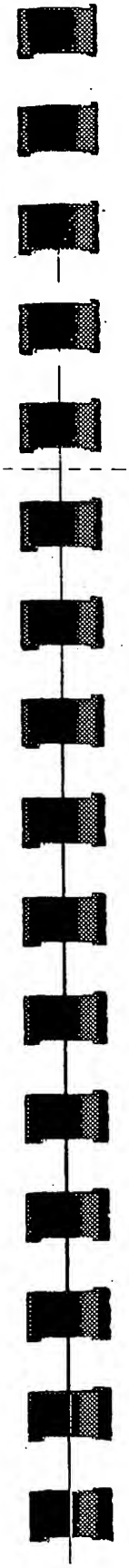
Vir gene induction is followed by the appearance of single-stranded nicks within the 25 bp border sequences which flank the T-DNA (Stachel and Zambryski, 1986; Albright *et al.*, 1987) and the appearance of a single-stranded linear molecule which corresponds to the T-DNA (Fig. 1.2). The products of the *vir*D operon are thought to be responsible for this specific endonuclease activity (Yanofsky *et al.*, 1986). By a mechanism which remains unknown, although thought to be analogous to bacterial conjugation, the T-DNA is transferred to the plant cell and stably inserted into the nuclear DNA (Stachel and Zambryski, 1986), an event possibly involving proteins coded for by the *vir*E operon (Winans *et al.*, 1987). A model (after Lichtenstein, 1986) depicting the early molecular events in the interaction between plant and *Agrobacterium* during crown gall formation is shown in Fig. 1.2.

1.1.5 *Agrobacterium* plasmids as transformation vectors

The natural ability of *Agrobacterium* to transfer defined sequences of DNA into the plant genome has been exploited in the development of a variety of plant transformation vectors. These vectors capitalize on several inherent characteristics of the *Agrobacterium*-mediated transformation process. During the early 1980s several research groups engineered Ti plasmids to remove all the T-DNA *onc* genes. The first important discovery was that the *onc* genes encoded by the Ti plasmid are neither required for the transfer of the T-DNA to the plant cell, nor its integration into the nuclear DNA. Hence, these genes can be replaced, not only allowing the insertion of foreign DNA, but also removing the *onc* functions. However, it should be noted that *nos* or *ocs* are useful marker genes for transformation because the enzyme activity of their gene products can be detected by a simple assay. To date a limit on insert size has not been reported. The third important milestone was the discovery that the *vir* gene products can also function in *trans*. Finally, non-oncogenic T-DNAs present in regenerated whole plants are transmitted to progeny in a Mendelian fashion.

1.1.6 Basic components of non-oncogenic Ti plasmid vectors

Taking the characteristics of gene transfer mediated by *Agrobacterium* into account, any foreign DNA that has been cloned can be transferred into the genome of a dicotyledenous plant cell. The foreign DNA to be transferred must be flanked by the T-DNA border sequences and stably maintained in an *Agrobacterium* strain harbouring a full complement of *vir* genes, either in *cis* or in *trans* (located on a separate virulence 'helper' plasmid). It should be mentioned here that *Agrobacterium* chromosomal genes associated with virulence have also been



described and are thought to be involved with the bacterial recognition of a component of the plant cell surface and subsequent attachment.

The removal of *onc* functions means that transformed tissues are no longer recognizable as neoplastic outgrowths which can be selected by their ability to grow on a medium lacking phytohormones. To resolve the problem of identifying transformed cells, bacterial antibiotic-resistance genes have been placed under the control of T-DNA promoters and polyadenylation signals and inserted between the 25 bp repeat sequences. Such chimaeric antibiotic-resistance genes are efficiently expressed in plant cells in a dominant fashion in any genetic background. It is useful to have marker genes tightly linked to the foreign DNA for two reasons; firstly, so that direct selection of transformed plant tissue can be carried out to ensure that the foreign DNA is transferred, and secondly, as a guarantee that the flanking foreign DNA in the particular transformed clone selected has not been inserted into a region of the genome that is not transcribed.

1.1.7 General purpose non-oncogenic plant transformation vectors based on the Ti plasmid

Both *A. tumefaciens* and *A. rhizogenes* have been used to transfer foreign DNA to plants. Much less is known concerning the genetic functions of the Ri plasmid and so transformation vectors involving *A. rhizogenes* will be discussed separately (1.1.8). A wide variety of general purpose transformation vectors based on the Ti plasmid have been developed (see Tables 1.1 and 1.2 for examples). These do not contain any oncogenic sequences and hence normal plant growth can be obtained following the transfer of DNA into the nucleus of the plant cell. Non-oncogenic vectors that are currently in use can be divided into two types, *cis* or *trans*, depending on whether the T-DNA regions, flanked by the 25 bp direct repeat sequences, are carried on the same replicon as the *vir* genes or on a separate plasmid (Fig. 1.3). The former (*cis*-acting *vir* genes) are often referred to as co-integrative vectors; whilst the latter, with *trans*-acting *vir* genes, are commonly called binary vectors.

1.1.7.1 *Cis* vectors

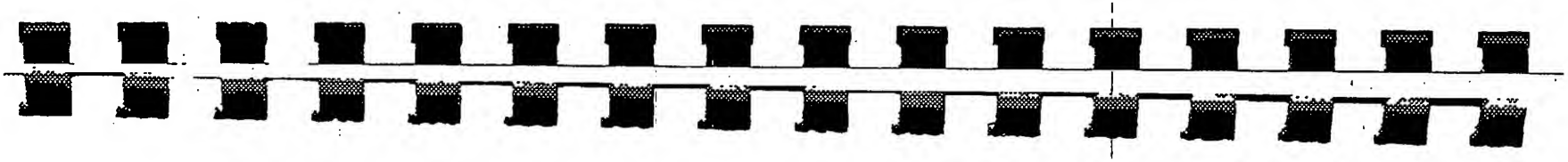
These are derivatives of wild-type Ti plasmids in which the T-DNA *onc* genes have been removed and, in some cases, replaced by a specific piece of DNA which has a region of homology to a small cloning vector that can replicate only in *E coli*. This vector strategy depends on co-integration in *A. tumefaciens* between homologous regions on the modified Ti plasmid (*vir* helper) and a small *E coli* cloning vector (intermediate vector) which contains a selectable marker gene that will function in plant cells and unique sites for the insertion of foreign DNA.

The intermediate vector containing foreign DNA sequences is normally introduced into the *A tumefaciens* by conjugation and, using appropriate selection, transconjugants can be obtained in which the foreign DNA has been stabilized within the T-DNA as a result of homologous recombination (Fig. 1.3A).

Table 1.1 Example co-integrative-type Ti plasmid vectors (*cis*-acting *vir* genes)

Cloning (intermediate) vector (size)	Co-integration host *vir* plasmid	Homology region	Ori	Mob/Tra helper plasmids	Bacterial selection marker	T-DNA border	Plant selection marker	Nos/ ocs	Cloning sites and comments
pMON200[1] (9.5 kb)	pTiB6S3-SE[1] (GV3111)	LIH	pBR322 (Col E1)	pR64drdll pGJ23 (JM101)	Sm/Sp	Rb (SEV) pTiT37	nos-*npt-II*	Nos	Unique sites for *Eco* RI, *Hind* III, *Xba* I, *Xho* I
pMON273[2] (10 kb)	pTiB6S3-SE (GV3111)	LIH	pBR322	JM101	Sm/Sp	Rb (SEV) pTiT37	CaMV35S-*npt*-II	Nos	Unique site for *Hind* III
pMON316[2] (11 kb)	pTiB6S3-SE (GV3111)	LIH	pBR322	JM101	Sm/Sp	Rb (SEV) pTiT37	nos-*npt-II*	Nos	Unique sites for *Bgl* II, *Cla* I, *Kpn* I and *Eco* RI between CaMV-35S promoter and Nos poly(A) site*
pGV1103[3] (6.5 kb)	pGV3850[4] (C58C1)	Ap^r gene	pBR322	JM101	Km	None	nos-*npt-II*	—	*Eco* RI Duplicate pBR322 sequences between T-DNA borders
pGV831[5] (8.9 kb)	pGV2260[5] (C58C1)	Ap^r gene	pBR322	JM101	Sm/Sp	Rb/Lb pTiB6S3	nos-*npt-II*	Ocs	*Bam* HI Duplicate pBR322 sequences are not between T-DNA borders

1 Fraley *et al* (1985); 2 Saunders *et al.* (1987); 3 Hain *et al* (1985); 4 Zambryski *et al* (1983); 5 Debleare *et al* (1985).
*pMON316 is a useful expression vector.



pMON316 is a useful expression vector.

Table 1.2 Example binary vectors (*trans*-acting *vir* genes)

Cloning vector (Size)	Virulence helper plasmid*	Wide host range origin	Host strain	Bacterial selection marker	Mob/Tra helper plasmids	T-DNA borders	Plant selection marker	Nos/ Ocs	Cloning sites and comments
pBin19[1] (10 kb)	pAL4404[2] (deletion mutant of pTi Ach5)	pRK252 (derived from pRK2)	HB101/ C58C1	Km	pRK2013 (HB101)	Rb/Lb pTiT37	nos-*npt-II*	—	Unique sites for *Eco* RI, *Bam* HI, *Hind* III, *Sst* I, *Sma* I, *Xba* I and *Sal* I IPTG + X-GAL screening
pAGS113[3] (16 kb)	pAL4404	pRK2	HB101/ C58C1	Km	pRK2013 (HB101)	Rb/Lb pTiA6/Ach5	nos-*npt-II*	—	*Cla* I, *Bam* HI
pAGS125[3] (17.6 kb)	pAL4404	pRK2	HB101/ C58C1	Km, Tc	pRK2013 (HB101)	Rb/Lb pTiA6/Ach5	nos-*npt-II*	—	*Cla* I, *Bam* HI
pARC8[4] (28 kb)	pRiA4*	pRK2	HB101/ A4	Tc, Ap†	pRK2013 (HB101)	Rb/Lb pTiT37	nos-*npt-II*	—	*Eco* RI, *Hind* III
Binary[5] (17 kb)	pAL4404	pKT240**	LE392/ C5851	Sm, Gm	pRK2013 (mm294)	Rb/Lb pTiT37	nos-*npt-II*	Nos	*Eco* RI, *Kpn* I, *Sma* I, *Xba* I, *Sal* I
pGA469[6] (10.8 kb)	pTiA6 pTiT37 pTiBo542	pTJS75 (derived from RK2)	K802/ Al36	Tc	pRK2073	Rb/Lb pTiT37	nos-*npt-II*	—	*Eco* RI, *Hind* III

1 Bevan (1984); 2 Hoekma *et al.* (1983); 3 Van den Elzen *et al.* (1985); 4 Simpson *et al* (1986); 5 Matzke and Matzke (1986); 6 An *et al.* (1985).

*pARC8 will also function with *A tumefaciens vir* helper plasmids.

**pKT240 replicon is stable in *Agrobacterium* without selection.

†Ap^r gene between T-DNA borders may be useful for plasmid rescue of T-DNA.

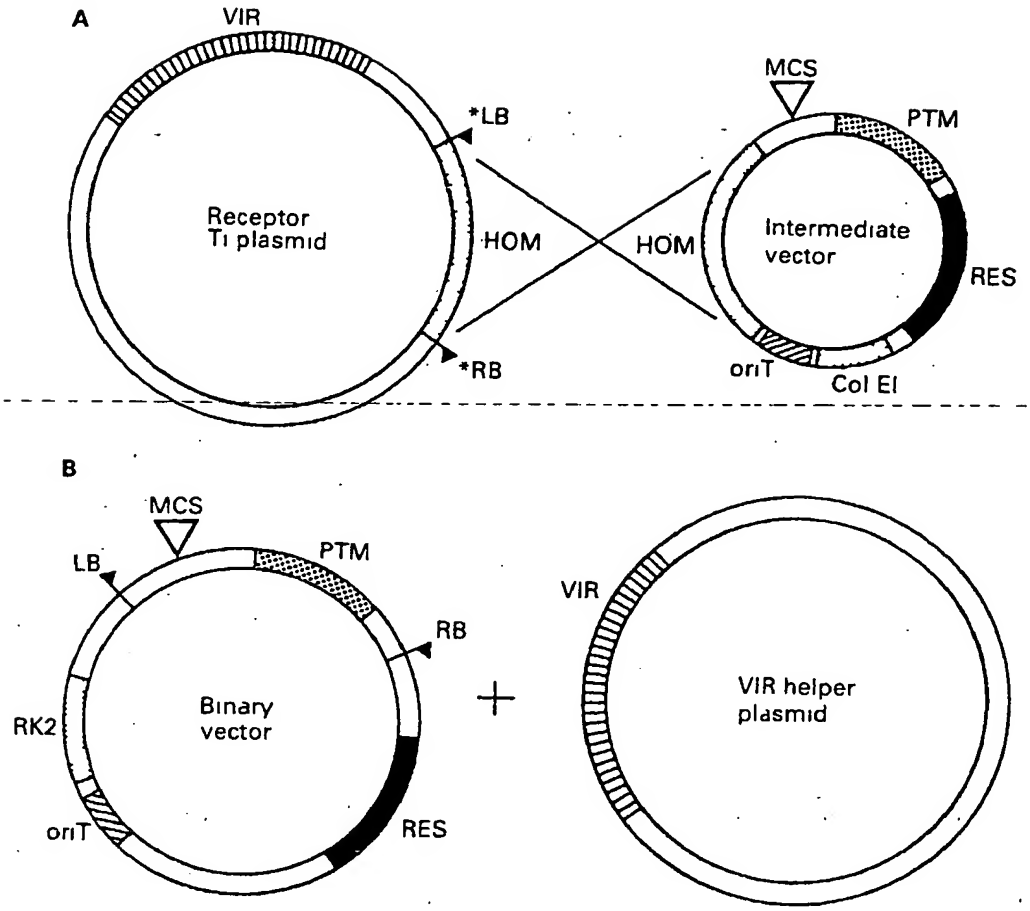


Fig. 1.3 *Schematic diagram of generalized co-integrative (A) and binary (B) vector systems* VIR = virulence region; HOM = homologous regions within which recombination may occur for co-integration. LB, RB = left and right borders (* either LB or RB or both may be present on the intermediate vector; see text); MCS = multicloning site, PTM = plant transformation marker; RES = antibiotic-resistance marker to select for presence of vector sequences in bacterial host, oriT = origin of transfer and *bom* site for conjugative mobilization of vectors; Col E1 = origin of replication from plasmid Col E1, RK2 = wide host range origin of replication from plasmid pRK2

In some vectors such as pGV3850 (Fig. 1.4) both T-DNA borders are on the modified Ti plasmid. In vectors such as pGV2260 (Debleare *et al.*, 1985) the 25 bp repeat sequences are on the intermediate vector, whilst in pTiBS3-SE (split-end vector; Fraley *et al.*, 1985) the right border is on the intermediate vector and the left T-DNA border is on the *vir* plasmid. Wherever the initial positions of the 25 bp repeat sequences, the net result after co-integration is the insertion of foreign DNA sequences between the T-DNA border repeats on a modified Ti plasmid. Thus, in *A. tumefaciens* strains containing these constructs, the T-DNA is transferred to the plant genome during transformation as a result of the *vir* region acting in *cis*.

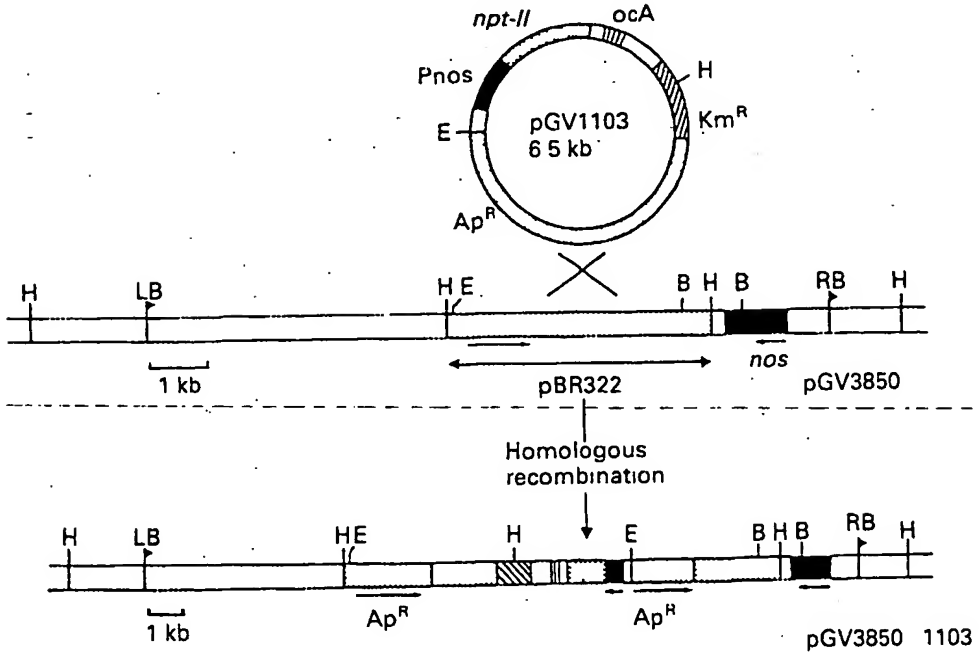


Fig. 1.4 *Structure and use of the co-integrative vector pGV3850.* Maps showing the structure of the plant transformation vector pGV3850 and the intermediate vector pGV1103, and a possible outcome of co-integration of pGV1103 into pGV3850. LB, RB = left and right borders, Pnos = nopaline synthase promoter; *npt-II* = the neomycin phosphotransferase-II coding sequence from Tn5; ocA = octopine synthase polyadenylation site; ⁻ = pBR322-derived regions of homology between the plant transformation and intermediate vectors; E = *Eco* RI, H = *Hind* III, and B = *Bam* HI restriction sites; *nos* = nopaline synthase, Ap^R and Km^R = ampicillin- and kanamycin-resistance genes.

To give one example in detail: pGV3850 (Zambryski *et al.*, 1983) is a *cis* vector in which the *onc* genes of a nopaline-type Ti plasmid (C58) have been deleted and replaced by pBR322 (Fig. 1.4). Any foreign DNA sequence which has been cloned in pBR322 can be introduced into pGV3850 by a two-step process of transfer into the *Agrobacterium* via conjugation followed by recombination. In order to carry this out, pBR322 containing the gene sequence to be transferred to the plant cell and a resistance marker which allows selection in *Agrobacterium* (kanamycin or streptomycin/spectinomycin) is introduced into an *E. coli* strain (GJ28) containing two helper plasmids, pGJ28 and pR64*drd*11. These plasmids provide Col E1 helper functions which allow transfer of all three plasmids via conjugation into *Agrobacterium*. However, pBR322 is unable to replicate in *Agrobacterium*, and hence will not be maintained; nevertheless, recombination can take place between pBR322 and pGV3850 which results in the transfer of the gene sequence of interest into the Ti plasmid. Transconjugants can be selected for by the resistance encoded by the plasmid and resistance to rifampicin which is encoded by the chromosome of *Agrobacterium*. Fine mapping of the insert within pGV3850 is carried out by Southern analysis.

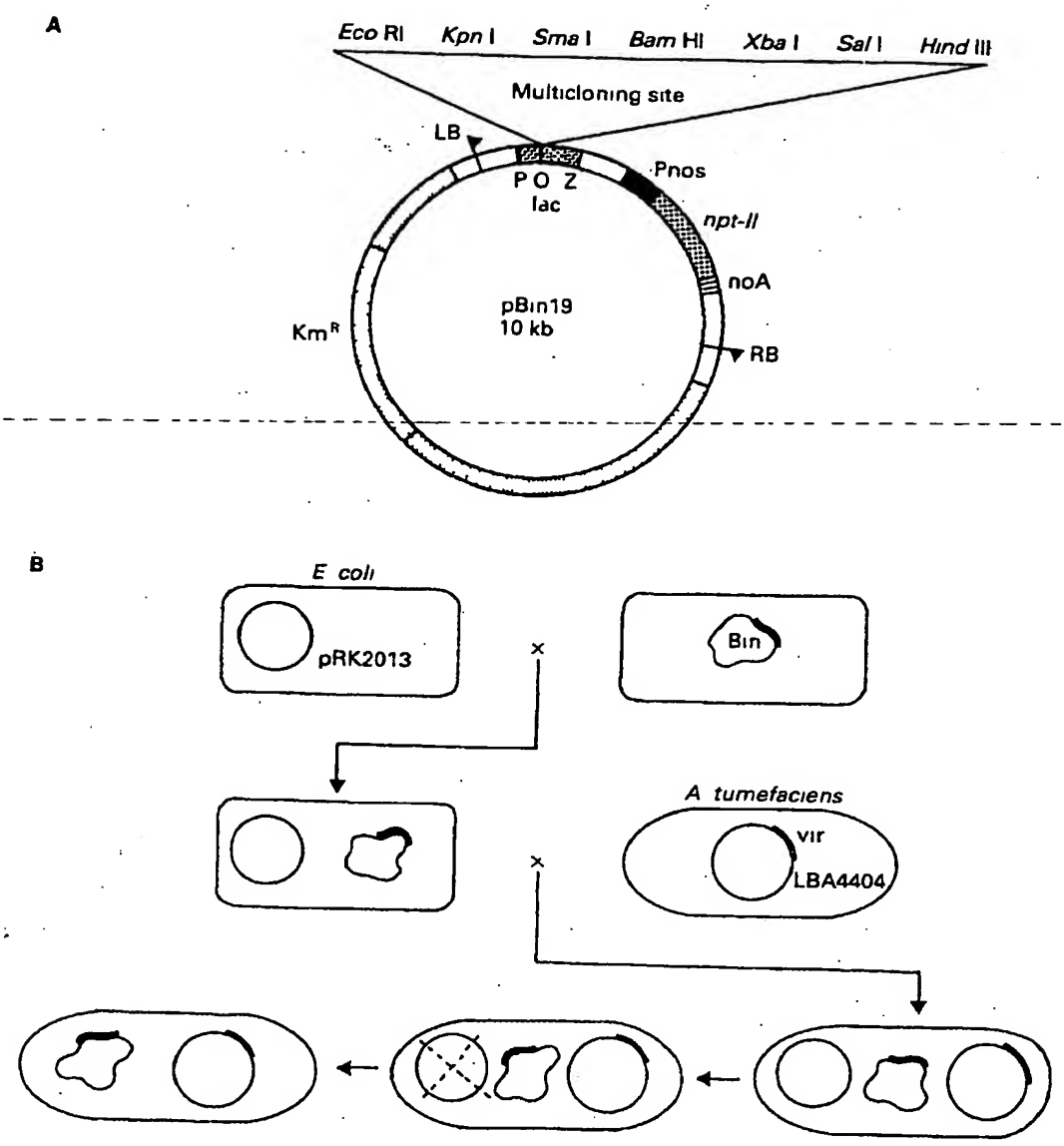


Fig. 1.5 *Structure and use of the binary vector pBin19*
A Map of the binary vector pBin19. LB, RB = left and right borders; lac = α-complementary region of lac operon; Pnos = nopaline synthase gene promoter; *npt-II* = neomycin phosphotransferase-II coding sequence from Tn5; noA = nopaline synthase gene polyadenylation site; ⋱ = pRK252-derived sequences containing the RK2 wide host-range origin of replication.
B Mobilization of pBin19 into *A tumefaciens* LBA4404 using the helper plasmid pRK2013, and subsequent elimination of the helper plasmid in *Agrobacterium*

1.1.7.2 *Trans* vectors

Trans or binary vectors are based on plasmids that can replicate in both *E. coli* and *Agrobacterium* and which contain the T-DNA border sequences (Fig. 1.3B). These can be designed so that the border sequences flank multiple cloning sites which allow insertion of foreign DNA, and markers that allow direct selection of transformed plant cells. The plasmids can be manipulated in *E. coli* and transferred via conjugation to *Agrobacterium* strains which contain a Ti plasmid which bear a *vir* region, but lacks T-DNA and 25 bp repeat sequences. Such plasmids are often simply deletion mutants of wild-type octopine or nopaline-type Ti plasmids (Table 1.2). Transfer of the foreign DNA on the cloning vector to the plant cell can be mediated by the activity of the *vir* region functioning in *trans*.

pBin19 is a commonly used binary vector based on the wide host range replicon of pRK252 (Fig. 1.5A). Hence it can replicate in both *E. coli* and *Agrobacterium*, allowing the insertion of foreign DNA into the vector followed by screening in *E. coli* prior to transfer into *Agrobacterium*. The vector contains a kanamycin-resistance (Km^r) marker for direct selection in bacteria as well as T-DNA border sequences flanking a dominant selectable marker for use in plant cells (a kanamycin-resistance gene flanked by the *nos* promoter and poly(A) addition site) and a multiple cloning region within the α-complementary region of β-galactosidase. Screening of recombinants can be carried out in Lac^- *E. coli* via α-complementation in the presence of IPTG and X-GAL (Gronenborn and Messing, 1978). The vector can be mobilized into *Agrobacterium* by conjugation using the helper functions of pRK2013. The *Agrobacterium* host, LBA4404, contains a Ti plasmid (pAL4404) from which the T-DNA has been deleted, but which contains an intact *vir* region. Transconjugants containing pBin19 can be selected for by resistance to kanamycin and rifampicin.

Examples of vector systems having *trans*-acting *vir* genes are presented in Table 1.2. These binary vectors vary in terms of size, stability of replication in *Agrobacterium*, cloning sites available for insertion of foreign DNA, α-complementation to allow screening of recombinant plasmids on X-GAL plates, marker gene(s) for selection of transformed plants and marker genes for selection of transconjugants, but most are known to be compatible with several *A. tumefaciens vir* helper plasmids and many also with Ri plasmids.

1.1.8 Plant transformation vectors utilizing *A. rhizogenes*

These have been primarily used in the transformation of plant types where the regeneration of whole plants from single cells has proved difficult. In a number of species, plant regeneration can occur from root cultures induced by *A. rhizogenes* through somatic embryogenesis or organogenesis. The mechanism by which the hairy root phenotype is suppressed to allow shoot morphogenesis is not presently understood. Plants regenerated from hairy roots are often morphologically altered with crinkled leaves, a plagiotrophic root system, stunted growth, and poor fertility (Tepfer, 1984).

1.1.8.1 *Cis* vectors

Here an intermediate vector is constructed which allows manipulation in *E. coli* and contains a region of the T-DNA of the Ri plasmid. Introduction of the vector into *A. rhizogenes* containing a normal Ri plasmid, results in the stabilization of the sequences of the intermediate vector as a result of intramolecular homologous recombination within the Ri plasmid (Jensen *et al.*, 1986).

1.1.8.2 *Trans* vectors

Disarmed binary vectors derived from *A. tumefaciens* (e.g. Bevan, 1984; Van den Elzen *et al.*, 1985) may be introduced into *A rhizogenes* strains and will replicate stably, as long as selection is maintained. Subsequent infection of the plant by *A. rhizogenes* carrying a binary vector causes transfer of the T-DNA from the binary vector, in addition to the T-DNA of *A. rhizogenes*, into the plant genome (Shahin *et al.*, 1986; Simpson *et al.*, 1986). The genes to be transferred to the plant genome are located between the binary vector T-DNA border sequences which operate in *trans* after induction of the *vir* region of the resident wild-type Ri plasmid (Simpson *et al.*, 1986; Trulson *et al.*, 1986; Sukhapinda *et al.*, 1987). This may be due to the large degree of homology, at the DNA level, between the virulence regions of *A tumefaciens* and *A. rhizogenes* (Huffman *et al.*, 1984).

Tomato and cucumber hairy roots have recently been induced by a wild-type *A. rhizogenes* strain harbouring a binary vector with a Km^r selectable marker. Regenerants from these kanamycin-resistant hairy root cultures were screened for plants with a normal phenotype and several were recovered which lacked the T-DNA of *A. rhizogenes* but possessed the T-DNA of the binary vector (Shahin *et al.*, 1986; Trulson *et al.*, 1986) presumably due to the independent transfer of the separate T-DNAs. This feature of *A. rhizogenes* makes it attractive as a transformation vector if the process can be extended to more crop plants.

1.1.9 Specialist uses of transformation vectors based on the Ti plasmids

Tables 1.1 and 1.2 are by no means an exhaustive list, but do give an idea of the range of properties associated with some of the more widely used general transformation vectors. However, there are now available various plant transformation vectors designed for specific purposes (Fig. 1.6).

Expression vectors have cloning sites which enable the easy creation of transcriptional fusions with promoters known to function in plant cells. These are used to study the gene products of eukaryotic cDNA clones, bacterial genes, or mutated coding sequences in higher plants. Such vectors include pAP2034 (Velten and Schell, 1985), pMON316 (Sanders *et al.*, 1987), pA10C3 (Bevan *et al.*, 1985).

Similarly, vectors have been developed which have promoterless 'reporter' genes with a 5′ multilinker for the insertion of promoter sequences, an example being the promoter probing vehicle (pGA482) described by An (1986).

Fig. 1

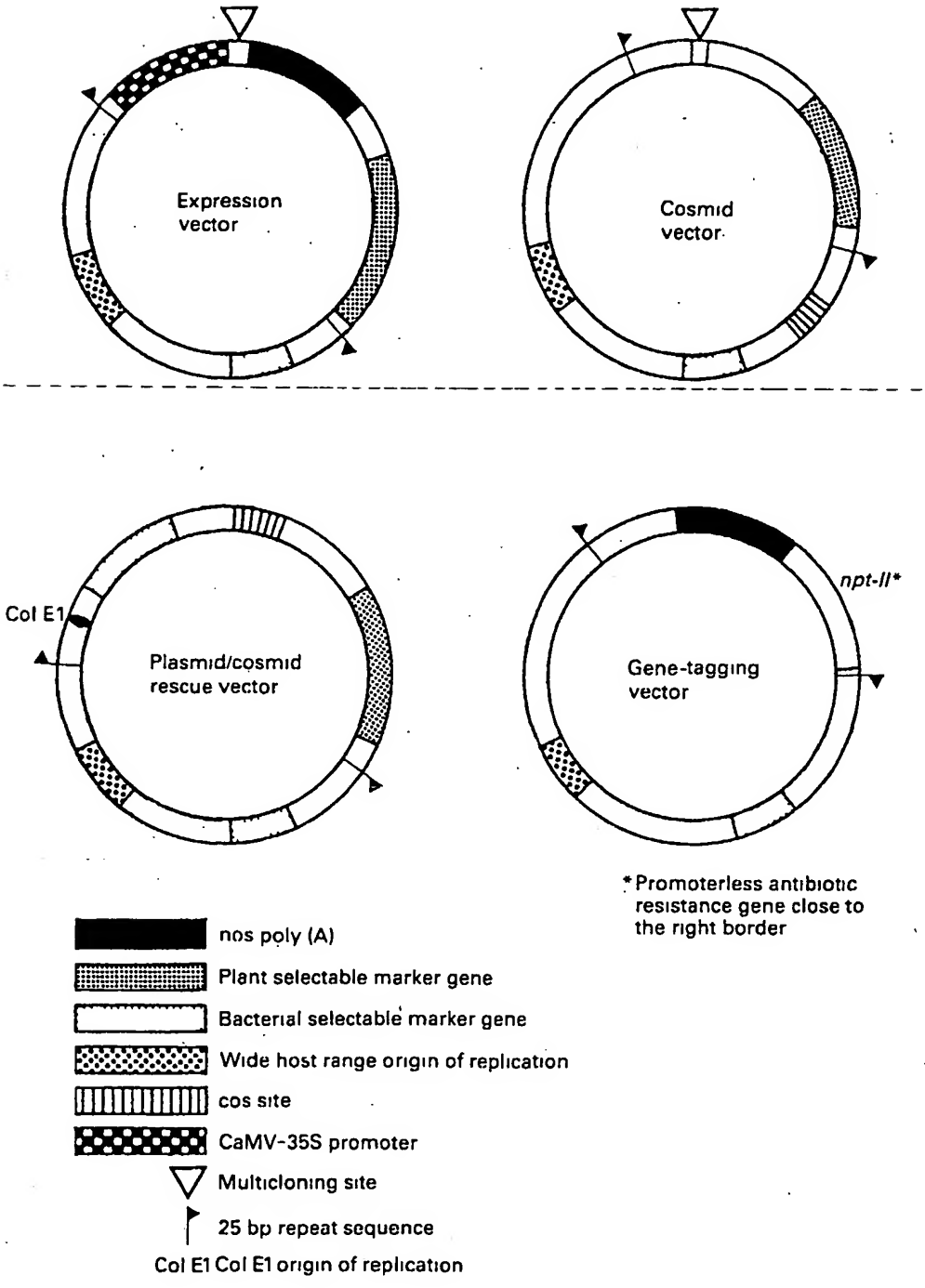


Fig. 1.6 *Schematic diagram of specialized Ti plasmid-based plant transformation vectors*

Cosmid vectors have a *λ cos* site outside the T-DNA region, thus allowing packaging of plant transformation vectors carrying large DNA inserts. Examples of this type of vector include pAGS127 (Van den Elzen *et al.*, 1985) and many of the vectors in the pCV001-311 family described by Koncz and Schell (1986).

Plasmid/cosmid rescue vectors have bacterial antibiotic-resistance genes and/or an *E. coli* replication origin, or a *λ cos* site between the T-DNA borders and can facilitate the cloning of flanking sequences at T-DNA integration sites by plasmid or phage rescue techniques. Such vectors include pGV3850 (Zambryski *et al.*, 1983), pEND4K (Klee *et al.*, 1985) and pGA471 (An *et al.*, 1985) and are useful for insertion mutagenesis approaches to gene cloning and in the study of genome position effects on gene expression. A further type of vector in this category is pC22 which can be used for the construction of genomic libraries in *E. coli* for transfer to *Agrobacterium* and then to large populations of plant cells. Attempts to use this vector in a genetic complementation approach to gene cloning in higher plants are currently underway by Simoens *et al.* (1986).

Gene-tagging vectors contain promoterless selectable marker genes close to the right T-DNA border. Random integration of such T-DNA in the proximity of a plant genomic DNA sequence with promoter activity restores the activity of the marker gene, thus allowing the selection of this particular type of transformant on media containing antibiotics. Such vectors are therefore used to clone sequences with promoter activity (Andrè *et al.*, 1986).

1.1.10 Choice of *Agrobacterium* Ti plasmid vector system

The choice of vector system for a particular transformation procedure depends on many factors, not the least of which are the aim of the experiment and the particular species involved. It is obvious from the preceding section that vectors can be designed to perform specialist functions. However, it is important to realize that no one vector system can be used in every circumstance, particularly with regard to host range, as many were originally tested using only model plant transformation systems, often tobacco or petunia, and almost without exception a solanaceous species. It is proposed to outline some of the properties of general transformation vectors that should be considered when attempting to set up a new plant genetic transformation system.

1.1.10.1 *Cis*- vs. *trans*-type vectors, conjugation and plasmid stability

The intermediate vector components of co-integrative (*cis*-type) vectors and modern binary vectors are relatively similar in size (6.5–28 kb) and are generally easily isolated from *E. coli*. Thus, all recombinant DNA manipulations may be carried out in *E. coli* without any problems. Mobilization of such vectors into *Agrobacterium* is generally not difficult but there are differences in mobilization frequences depending on the choice of vector (Koncz and Schell, 1986). Systems

for the conjugational transfer of cloning vectors between *E. coli* and *Agrobacterium* are specific to plant transformation vectors and are therefore discussed in detail in 1.10. It should also be noted that apart from mobilization efficiency, the frequency of transconjugant selection in *Agrobacterium* is much higher for binary vectors as co-integration is not required (Fig. 1.3). In addition it is easy to conjugate and stabilize a binary vector in any new *Agrobacterium* strain in order to place the vector in combination with different *vir* systems, chromosome backgrounds or bacterial phytohormone production determinants, whereas to achieve the same result with co-integrative vectors often requires the construction of a new *vir* helper receptor plasmid.

To date, the wide host range replication functions most convenient to use (in terms of the size of the replicon), and which allows stable maintenance of binary vectors in *Agrobacterium* under selection, are derived from plasmid pRK252. The replication origin from pKT240 is also reasonably small, functions in both *Agrobacterium* and *E. coli* (Matzke and Matzke, 1986) and is claimed to be stable in the absence of selection.

Several copies of binary vectors are often found in *Agrobacterium*, whilst *trans*-type vectors are generally only present as a single copy plasmid. This difference in copy number between the two systems has not been conclusively proved to have any effect on transformation efficiency, copy number of T-DNA inserts or organization of T-DNA within transformed plant cells.

1.1.10.2 *Vir* genes, 25 bp repeat sequences and plant host range

It has been known for some time that some wild-type *Agrobacterium* strains are more virulent on certain dicotyledenous species than on others (for example see Byrne *et al.*, 1987). This effect is thought to have little to do with the oncogenicity of the T-DNA involved, but instead relates to the efficiency of the particular *vir* genes in the T-DNA transfer process, the function of Ti plasmid regions outside the *vir* and T-DNA sectors which enhance the transfer process, and the chromosomal background of the *Agrobacterium* strain utilized. This host range phenomenon is also apparent when *vir* genes from the same wild-type Ti plasmid are used to drive T-DNA transfer in non-oncogenic vectors.

Vir systems

As far as it is known, all wide host range *vir* gene systems are able to recognize 25 bp repeat sequences which flank both Ti and Ri T-DNA regions and are able to effect the excision the T-DNA and its transfer to the plant genome (Hoekema *et al.*, 1984; Klee *et al.*, 1985; Simpson *et al.*, 1986). However, although the virulence systems of different *Agrobacterium* strains share common functions they are not identical and some may give rise to more efficient transformation on some plant species than on others (e.g. Byrne *et al.*, 1987; Fillatti *et al.*, 1987). The common *vir* systems used in conjunction with non-oncogenic plant transformation vectors are shown in Tables 1.1 and 1.2. In general, most binary vector

systems have utilized an octopine-type plasmid (pTiAch5) with the T-DNA and 25 bp repeat sequences deleted (pAL4404). The co-integrative receptor plasmids pTiB6S3-SE and pGV2260 have been developed from the octopine-type Ti plasmid pTiB6S3, whilst pGV3850 is based on the nopaline-type plasmid pTiC58. Thus, any difficulty in obtaining transformants when using binary vectors in LBA4404 may reflect, in many cases, the limitations of the pAL4404 *vir* functions.

It has recently been shown that normal transformed shoots lacking T-DNA oncogenes can be regenerated from plant tissue where wild-type *A. tumefaciens* (Fillatti *et al.*, 1987) and *A. rhizogenes* (Shahin *et al.*, 1986; Trulson *et al.*, 1986) strains have been used to provide *vir* helper functions for non-oncogenic binary vectors carrying dominant marker genes. This data suggests that binary vector T-DNA can be transferred to some cells independently of the Ri or Ti plasmid T-DNA. Recently, a supervirulent Ti plasmid has been described (pTiBo542) which is found in the wild-type *A tumefaciens* strain A281 (Komari *et al.*, 1986). Compared to other strains of *A. tumefaciens*, A281 induces large, fast-appearing tumours on a wider range of host plants. When this wild-type plasmid was used as a *vir* helper for a binary vector it was found to give rise to at least a 3-fold increase in transformation efficiency when compared to other *vir* systems (An *et al.*, 1985; An, 1985). The use of 'supervirulent' wild-type Ti and Ri plasmids may have an important role to play in the future development of crop plant transformation systems.

T-DNA transmission enhancers

In the wild-type plasmid pTiA6NC, a specific sequence flanking the right-hand 25 bp repeat sequence (called *overdrive*) has been claimed to enhance T-DNA transmission (Peralta *et al.*, 1986). DNA sequences at similar sites with an almost identical core sequence (TGTTTGTT) have also been found on a nopaline Ti plasmid (pTiT37) and an Ri plasmid (pRiA4). Thus, it may be important to check that such sequences are flanking right T-DNA border sequences in the vector to be used.

Chromosomal background of Agrobacterium strain

It should also be remembered that the genetic background of the host *Agrobacterium* strain should also be taken into account, as some *vir* functions, mainly concerned with plant surface recognition and bacterial binding, are chromosomally located (Douglas *et al.*, 1985; Gurlitz *et al.*, 1987). Individual strains of *Agrobacterium* also exhibit differences in growth rates, polysaccharide production and general toxicity to plant cells, which can all effect the efficiency of transformation *in vitro*.

Phytohormone production by Agrobacterium

It has been known for several years that *Agrobacterium* strains secrete both auxins (IAA) and cytokinins (*trans*-zeatin), and such activities may produce a

local hormonal environment which can exert physiological effects on plant cells in terms of cell competence for transformation, transformed cell growth rate and exhibition of morphogenesis. For example, nopaline strains have a locus (*tzs*) just inside the boundary of the *vir* region which codes for an enyme responsible for constitutive *trans*-zeatin production by the bacterium (Beaty *et al.*, 1986). It has been shown recently that explant inoculation with the wild-type oncogenic nopaline strain C58 (pTiC58) enhanced regeneration from *Populus* leaf explants *in vitro* over that exhibited by the uninoculated controls. This effect was not seen when *Populus* leaf explants were inoculated with LBA4404, an octopine strain with the chromosomal background of Ach5 (Fillatti *et al.*, 1987).

Thus, when working with a particular plant species, or even varieties of the same species, it should be emphasized that a transformation vector may produce transformants more efficiently when driven by one type of *vir* region than any other in different chromosome backgrounds. Optimal combinations of *vir* systems, virulence enhancer functions and chromosomal background which give rise to efficient T-DNA transfer for any particular variety can only be determined by empirical testing.

1.1.10.3 Selectable/screenable marker genes for the identification of transformed plant cells

Selectable marker genes

Two main aspects of the marker gene have to be considered: firstly, its structure (nucleic acid sequence), which will determine factors such as regulation of transcription (constitutive, environmental or developmental expression), rate of transcription, transcript stability and efficiency of translation; secondly, the gene product itself, which is obviously responsible for the dominant expression of a suitable selective phenotype. The selectable functions on most general transformation vectors are prokaryotic antibiotic-resistance enzymes which have been engineered to be expressed constitutively in plant cells (Table 2.1). In some experiments, enzymes affording protection against specific herbicides have also been used successfully as dominant marker genes. The enzyme coding sequence is normally fused to promoters isolated from T-DNA or the CaMV genome at the 5′ end, and a polyadenylation signal, often again from a T-DNA gene, at the 3′ end. The most commonly used marker genes are those affording resistance to antibiotics such as kanamycin, G418 (e.g. Herrera-Estrella *et al.*, 1983; Bevan, 1984), hygromycin (Van Den Elzen *et al.*, 1985) and bleomycin (Hille *et al.*, 1986). Genes giving resistance to herbicides such as glyphosate have also been used recently as dominant markers for transformed cells (Shah *et al.*, 1986). As transformation selection marker genes function in plant cells and do not really affect recombinant DNA manipulations with transformation vectors, the use of such marker genes is discussed in detail in Chapter 2. To be of use, the selective agent concerned must be able to exert a stringent selection pressure on the plant tissue concerned. Kanamycin resistance has proved a very useful transformation marker and so all recombinant DNA manipulations and transformation experiments will be described using vectors carrying a Km^r gene.

Screenable marker genes

Screenable marker genes (Table 2.1) are included on many transformation vectors for two reasons. Firstly, to allow independent verification of the transformed status of tissues growing on media containing selective antibiotics or herbicides. Secondly, as a principal means of identifying transformants in conditions where transformation frequencies are high, and the transformed tissues are mainly of clonal origin; examples being transformed shoots and roots, and colonies derived from protoplasts plated at low density, which are normally considered to have developed from single transformed cells.

In the case of screenable markers, the ease, expense and specificity of the detection methods are important considerations. The assays currently available for the more commonly used screenable markers (chloramphenicol acetyl transferase, octopine and nopaline synthase, β-glucuronidase, β-galactosidase and luciferase) are all discussed in detail in Chapter 2 (Table 2.1). The production of agropine or mannopine is commonly used as a marker for oncogenically transformed cells in vector systems utilizing wild-type Ri plasmids.

1.1.10.4 Cloning sites in plant transformation vectors

In the present chapter, it will be assumed that the aim of the experiment will be simply to insert a previously cloned gene into a general plant transformation vector in *E. coli* and then conjugate it to *Agrobacterium* ready for transformation. The recombinant DNA, bacterial transformation and conjugation procedures, plasmid isolation and analysis techniques may be used generally with all types of plant transformation vectors.

Many transformation vectors have only one or two suitable restriction enzyme sites useful for cloning purposes, whilst others are more versatile and contain many useful sites on a multilinker, sometimes placed within a β-galactosidase α-complementation region to allow the visualization of recombinant transformed bacterial colonies on X-GAL/IPTG plates (Tables 1.1 and 1.2).

1.1.11 Cloning into plant transformation vectors

The basic recombinant DNA procedures required to insert genes into a plant transformation vector are no different to those used with any other type of small plasmid that will replicate in *E. coli*. A DNA restriction fragment carrying a gene of interest is either ligated directly into an intermediate vector (used for co-integration into *cis*-type Ti plasmid vectors) or a binary vector and then transformed into a suitable *E. coli* host strain by standard procedures.

The aim of the present chapter is to provide some simple guidance of how to approach the insertion of a DNA restriction fragment into plant transformation vectors, and is intended for the researcher with little experience in recombinant DNA technology. The manuals of Maniatis *et al.* (1982) and Boulnois (1987)

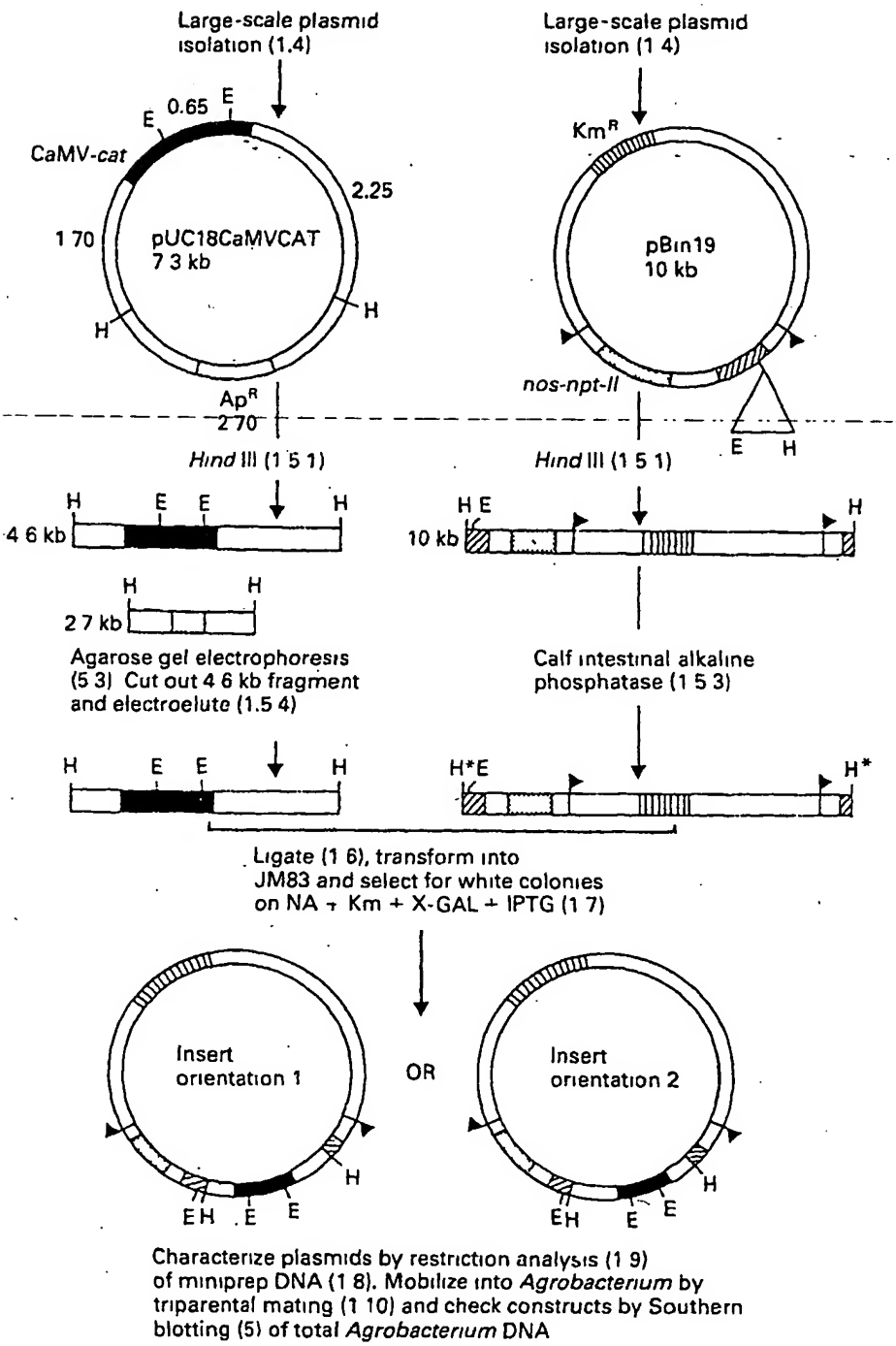


Fig. 1.7 *Cloning genes into pBin19* Schematic diagram of the cloning strategy adopted to insert the 4 6 kb *Hind* III fragment CaMV-*cat* from pUC18CaMVCAT into the *Hind* III site of pBin19.

should be consulted for further details on the principles of gene cloning and analysis of DNA sequences.

For convenience, throughout this section the binary vector pBin19 (Bevan, 1985), will be used as an example in all the recombinant DNA procedures (1.1.7.2 and Fig. 1.5). pGV3850 (Zambryski *et al.*, 1983) will be used as an example of a co-integrative vector (1.1.7.1 and Fig. 1.4). A flow diagram describing various stages of the insertion of foreign DNA into pBin19 is presented in Fig. 1.7.

The procedures used to insert genes into pBin19 are applicable to many other general plant transformation vectors, with the possible exception of the X-GAL/IPTG screening facility which is not available on all vectors. Apart from the obvious differences in the restriction enzyme sites used for cloning and antibiotic-resistance markers used to select for *E. coli* transformants and *Agrobacterium* transconjugants, it is important to match the DNA sequences required on the plant transformation vector for mobilization and transfer with appropriate conjugation helper plasmids (1.10). Other variations on the basic techniques may be required for some of the specialist vectors (1.1.9) and the reader is advised to consult original papers for details. Finally, it remains to be said that many of the plant transformation vectors, selectable and screenable marker genes, *vir* and conjugation helper strains mentioned in this manual are freely available. Interested researchers should apply for vectors and helper strains directly from the laboratories in which they were developed.

1.2 GENERAL MOLECULAR BIOLOGICAL PRACTICES

Good working practices help to ensure that enzymic reactions occur predictably and reliably, are kept free from contamination by other, non-specific nucleic acids, and that the integrity of desired DNA molecules is maintained through successive manipulations. Nucleases occur in significant quantities on the skin and therefore precautions must be taken to prevent these contaminating reactions. Some general points and procedures aimed at minimizing problems associated with nucleases, non-specific DNA and chemical contaminants are presented below:

- If glassware is used for the making and storing solutions, then it should be made of Pyrex, as soda glass contains leachable contaminants.
- Glassware and plastic centrifuge tubes should be acid-washed, soaked and scrubbed in the presence of a surface active cleaner such as Decon 90 (Decon Labs Ltd.), rinsed thoroughly in double distilled water, autoclaved at 121 °C for 15 min and dried before use.
- Pour off supernatants from bacterial cultures into waste beakers containing a disinfectant (e.g. Hycolin; William Pearson Ltd.). To recycle contaminated plastic and glass-ware, they should first be soaked in a disinfectant or autoclaved prior to washing. All disposable items contaminated by contact with bacterial suspensions or extracts should be sterilized before disposal.
- In addition to the washing described above, glassware which comes directly into

contact with RNA-containing solutions should be siliconized and baked in a dry oven at 180 °C for 2 h.

- Disposable plastic tips and microcentrifuge tubes should be handled with clean disposable gloves while packing into suitable dispensers and should be autoclaved and dried before use.
- Disposable gloves should be worn at all times when working at the bench.
- Water used to make up solutions should be of the purest grade available, and should be at least deionized and distilled.
- Chemicals used to make solutions should be at least analytical grade.
- In order to avoid cross-contamination and the introduction of RNase into reagents, spatulas should not be used when weighing out chemicals.
- Most solutions should be autoclaved before storage — those that need to be filter-sterilized are indicated in the text.
- Restriction and other DNA-modifying enzyme stocks should be stored at −20 °C and, when required for use, should be transferred immediately to an ice-bucket. They should be returned to −20 °C as quickly as possible.
- To ensure accuracy and reproducibility, Gilsons should be used carefully with a fresh tip for each pipetting operation and checked for accuracy at regular intervals.
- Two commonly used chemicals, phenol and ethidium bromide, are highly dangerous and should be used with great care. A spillage of phenol covering an area of skin the size of two hand prints can be fatal and thus it is important to have a solution of 7 : 3(w/v) PEG 3000/ethanol close at hand with which to swab the affected areas as soon as possible. Ethidium bromide is mutagenic and contact with skin should be avoided.
- Radioisotopes should be handled and disposed of according to local radiation regulations. Such regulations should be consulted prior to the design of any experiment utilizing radioisotopes and appropriate saftey measures incorporated into the procedure.

1.3 GROWTH AND MAINTENANCE OF BACTERIAL STRAINS

It is very important to be able to reliably maintain and select for specific strains of bacteria (Appendix 1 [IIA]) in order for results to be unambiguous. Described below are the basic bacteriological techniques required when constructing recombinant vectors for plant transformation. Most of the bacterial strains contain specific selectable markers, often carried extrachromosomally, and should be checked out regularly to guard against the possibility of contamination, mutation, or loss of specific plasmids. Many strains will be used frequently, whilst others will be used only occasionally; thus, methods are required both for the rapid generation of genetically pure bacterial colonies as inocula for larger cultures, and for the long-term storage of important strains. Selection media for commonly used bacterial strains are presented in Appendix 1 [IIB].

1.3.1 Materials

Equipment/consumables

- The majority of equipment and consumables required are presented in Appendix 1 [I].
- 2 ml screw-capped cryogenic storage tubes (Sarstedt).
- Microspatula (to be smaller in width than the internal diameter of the storage tubes).
- Small Dewar flask of liquid nitrogen.

Solutions

- Nutrient Broth (NB) (Oxoid): this medium should be made up as directed by the manufacturer, autoclaved and dispensed in the following types of containers:
  5 ml in Universal bottles for overnight cultures
  80 ml in 100 ml bottles for small-batch cultures
  400 ml in 500 ml bottles for large-scale cultures.
- Nutrient Agar (NA) (Oxoid): this medium should be made up as directed by the manufacturer, dissolved by steaming, dispensed in measured amounts in volumes convenient for pouring Petri dishes, and autoclaved.
- Antibiotic stock solutions (see Appendix 1 [IIB]).
- 40% glycerol in NB (autoclaved).
- X-GAL: dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BRL) at 40 mg/ml in dimethyl-formamide and store at −20 °C.
- IPTG: dissolve isopropyl-β-D-thiogalactopyranoside (Pharmacia) in sterile distilled water at 40 mg/ml and store at −20 °C.

1.3.2 Growth of overnight bacterial cultures

1 Using a flamed and cooled bacterial loop (heat until it glows red and then allow to cool to room temperature), transfer a single colony from a fresh bacterial plate containing selective antibiotics, to 5 ml of NB containing the same selective antibiotics in a Universal bottle.
NB: observe sterile procedures throughout!

2 (a) *E coli*: shake for 8–16 h at 37 °C using an orbital shaker with environmental control, a throw of 5–10 cm and a shake rate of 150–200 rpm.
(b) *Agrobacterium*: grow for 20 48 h at 28 °C using a similar shaker.

1.3.3 Streaking of bacterial cultures for single colonies

1 Dip a flame-sterilized bacterial loop into an overnight culture and streak out onto the surface of *dry* solid medium (with appropriate selective antibiotics)

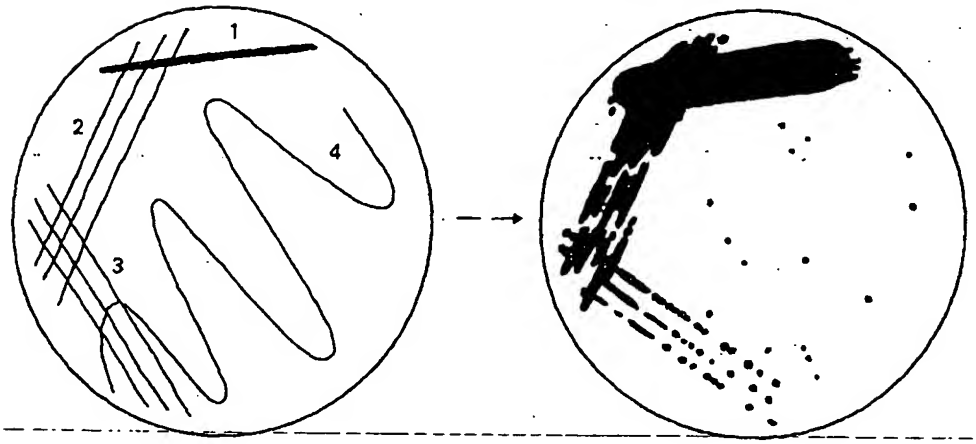


Fig. 1.8 *Streaking of plates to form single bacterial colonies* Bacteria streaked out in the order indicated. The loop need only be flamed and cooled between (1) and (2).

contained in a 9 cm Petri dish as indicated in Fig. 1.8. Flame the loop between the first and second streaks only.

2 If possible, slightly open the lid of the dish and allow to dry in a sterile flow cabinet for 10 min. Replace the lid and then incubate upside down for the same times and at the same temperatures given in 1.3.2.

3 Seal with Nescofilm and leave at room temperature if they are to be used within a couple of days, or store at 4 °C for up to a month.

1.3.4 Long-term storage of bacterial strains

1 Set up an overnight culture for the strain to be stored as in 1.3.2.

2 Mix 0.75 ml of overnight culture with 0.75 ml of 40% glycerol in growth medium in a 2 ml screw-capped cryogenic storage tube, label and flash-freeze by dropping into a Dewar flask of liquid nitrogen.

3 Store at −80 °C. The bacteria should remain viable for up to 10 years.

4 To recover rapidly growing cells from stored cultures, scrape off a portion of the frozen mixture using a flame-sterilized microspatula and resuspend in 5 ml of NB. Plate-out on bacterial plates containing selective antibiotics as described in 1.3.3.

1.3.5 Bacterial selection plates

Nutrient agar should be melted by steaming or use of a microwave and cooled to 45–55 °C (preferably in a suitable water bath) before adding antibiotics or other thermolabile components. Plates can be stored at 4 °C for several days to several

weeks, depending on the stability of the additives (see Appendix 1 [II] for details on antibiotics).

Apart from the inclusion of antibiotics to select for particular chromosomal backgrounds and maintenance of specific plasmids, many modern cloning vectors use combinations of colorimetric indicator substrates and inducers (such as X-GAL and IPTG) to screen for recombinant transformants. For example, the plant transformation vector pBin19 (1.6) contains a multicloning site for the insertion of DNA into a *lacZ* gene, and any insert at this site disrupts the production of a functional β-galactosidase enzyme. Therefore, transformed bacterial colonies harbouring recombinant plasmids are not able to degrade chromogenic substrates such as X-GAL and are white, whilst bacterial colonies containing plasmids without inserts are blue, owing to the cleavage of X-GAL by β-galactosidase.

Thus, for the screening of β-galactosidase production, add X-GAL and IPTG to cooled NA at a final concentration of 40 μg/ml. X-GAL/IPTG plates are not stable and should be made up freshly each time.

1.4 LARGE-SCALE PLASMID PREPARATION FROM *E. COLI*

1.4.1 Background

The isolation of small plasmids in large amounts from *E. coli* is an important part of any series of manipulations aimed at inserting genes into plant transformation vectors. Plasmids may contain the gene to be transferred on a specific restriction fragment, or may be part of the plant transformation vector system; either an intermediate vector for co-integration into a modified Ti plasmid, or a binary vector able to replicate in both *E. coli* and *Agrobacterium*.

The protocol presented below is one which has been used successfully with a wide range of plasmids (including pBin19, pBR322, pGV1103, pUC18) and is essentially a scaled-up version of a miniprep developed by Birnboim and Doly (1979). Separation of chromosomal and plasmid DNA is based on the observation that there is a limited pH range of 12.0–12.5 where linear chromosomal DNA is denatured but covalently closed circular plasmid DNA is not.

Cells are treated with lysozyme to weaken the cell wall and then lysed with SDS in the presence of NaOH. The ratio of cell suspension to alkaline SDS is important and should be strictly adhered to as this determines the final pH of the resulting mixture. On neutralization the chromosomal DNA re-anneals to form an insoluble clot, whereas the plasmid stays in solution. In addition, the high concentration of KOAc causes the precipitation of protein–SDS complexes and high molecular weight RNA. The plasmid is ethanol-precipitated from the supernatant and further purified by density gradient centrifugation. The crude DNA solution is mixed with ethidium bromide (EtBr) and caesium chloride (CsCl) and centrifuged until the ensuing CsCl density gradient reaches equilibrium. EtBr intercalates with DNA and decreases its density. As circular DNA is less able to unwind than linear DNA, plasmid molecules intercalate less EtBr and are

therefore denser than linear chromosomal DNA. Although bacteria have circular chromosomes, they are much too large to survive the isolation procedure intact and so plasmid DNA bands below chromosomal DNA on the CsCl gradient (Fig. 1.9). From 500 ml of original culture this method routinely produces 1–2 mg of plasmid DNA which is free from contaminating RNA and digests with all restriction enzymes so far tested.

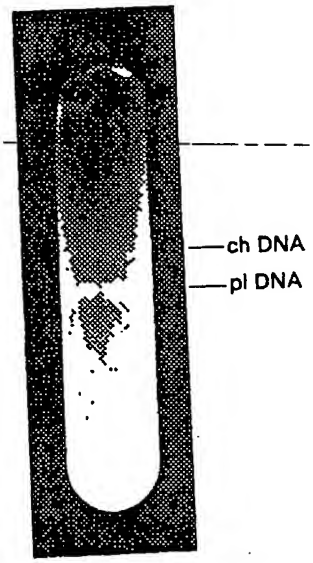


Fig. 1.9 *Banding of plasmid and chromosomal DNA in a CsCl density gradient*. Centrifuge tube after 22 h at 55 000 rpm photographed under UV light showing separation of chromosomal and plasmid DNAs

Isolated plasmid DNA exists in three forms, depending upon whether: (1) both strands are totally intact and the plasmid has its orginal supercoiled, covalently closed configuration (ccc); (2) one strand has been 'nicked' allowing the plasmid to relax to produce an open circle (oc); or (3) the DNA strand has been broken, resulting in a linear molecule. As a result of their different configurations, these three forms demonstrate different mobilities during agarose gel electrophoresis (Fig. 1.10). Faint bands migrating behind the oc form represent multimeric forms of the plasmid. A good plasmid preparation will contain mostly ccc and oc with very little linear DNA (Fig. 1.10, lane 3). An accurate determination of molecular weight can only be obtained from linear DNA; therefore when sizing a plasmid it must be linearized by restriction digestion before electrophoresis (Fig. 1.10, lane 2).

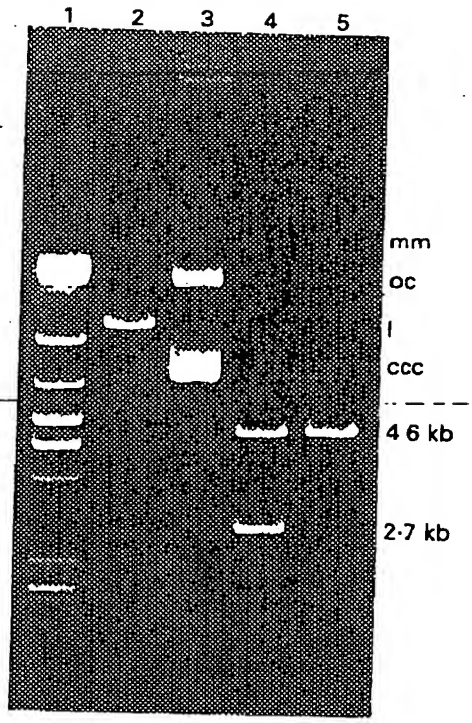


Fig. 1.10 *Preparation of DNA fragments for ligation*
(1) $\lambda \times$ *Hind* III + $\lambda \times$ *Hind* III + *Eco* RI markers, (2) pBin19 linearized with *Hind* III, (3) uncut density-gradient purified pBin19 showing ccc, l, oc and mm (multimeric) forms, (4) pUC18CaMVCAT cut with *Hind* III, producing 4·6 kb and 2·7 kb fragments, (5) 4.6 kb CaMV-*cat* fragment after isolation by electroelution

1.4.2 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1[I]).
- Refrigerated, medium-speed centrifuge (e.g. Sorval RC-5B, Dupont (UK) Ltd.) with 6 × 250 ml and 8 × 50 ml fixed angle rotors.
- 250 ml and 35 ml polyallomer centrifuge tubes with polypropylene screw-caps (e.g. appropriate Nalgene or LKB high-speed centrifuge tubes).
- Ultracentrifuge (e.g. Beckman L7-65 or equivalent) with high-speed, fixed-angle (e.g. Beckman Ti-80) or vertical (e.g. Beckman VTi-65) rotor.
- Polyallomer ultracentrifuge tubes and tube sealing device.
- Corex centrifuge tubes (Corning Medical) siliconized and autoclaved.
- Spectrophotometer with a UV range from at least 200–300 nm (e.g. Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer).
- Small plastic funnel plugged with polyallomer wool (Supa Aquatic Supplies Ltd.).

- Dialysis tubing.[d]
- Mediclips (Scientific Industries International Inc. (UK) Ltd.).
- 5 ml plastic disposable syringe (Monoject Division) fitted with a 0.4 cm × 19 gauge needle (Monoject Division).
- UV hand lamp (e.g. UV Products; Model UVGL-58) held in a clamp stand.
- Dark room.
- Waste beaker for EtBr/CsCl solution.
- CsCl (BDH; grade for ultracentrifuge gradients).

Solutions

- NB medium containing appropriate antibiotics:
  5 ml in Universals
  500 ml in a 2 litre conical flask.
- 2 M Tris-HCl pH 8.0 and pH 7.5. *For 1 litre*: dissolve 242.2 g of Tris-base in 800 ml of dH_2O and titrate to correct pH with concentrated HCl. Use a Tris pH electrode (Sigma). Make volume to 1 litre with dH_2O. These solutions are stable for several months at room temperature.
- Lysis solution (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 M glucose, 1 mg/ml lysozyme). *For 100 ml*:

2 M Tris-HCl pH 8.0	1.25 ml
0.5 M EDTA	2.00 ml
Glucose	9.01 g
Lysozyme (Sigma Grade 1)	0.10 g.

  This should be made up without lysozyme, autoclaved and stored at room temperature. Lysozyme should be added just before use.
- Alkaline SDS: (0.2 M NaOH, 1% SDS). *For 100 ml*:

10 M NaOH	2.0 ml
25% SDS	4.0 ml.

  Make up just before use by adding the components to 94 ml dH_2O.
- 3 M KOAc pH 5.2: dissolve 117.78 g of potassium acetate (BDH Analar) in 200 ml of distilled water. Titrate to pH 5.2 with glacial acetic acid (BDH Analar), make to 400 ml, autoclave and store at room temperature.
- 2 M NaOAc pH 5.6: dissolve 27.16 g of sodium acetate (BDH Analar, trihydrate) in distilled water. Titrate to pH 5.6 with glacial acetic acid (BDH Analar), make to 100 ml, autoclave and store at room temperature.
- TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5). *For 100 ml:*

2 M Tris HCl pH 7.5	0.5 ml
0.5 M EDTA	0.2 ml.

  Make to volume with dH_2O, autoclave and store at room temperature.
- 10 mM Tris-HCl pH 7.5 (make from 2 M Tris-HCl, pH 7.5; see above).

Cont.

- Ethidium bromide (Sigma) (5 mg/ml in distilled water): store at 4 °C in the dark. This solution is stable for several months.
- CsCl-saturated propan-2-ol: overlay a saturated CsCl solution with propan-2-ol, shake vigorously to emulsify and then allow phases to separate before use.
- Propan-2-ol (BDH Analar).
- 100% and 70% ethanol (BDH Analar).
- Diethylether (BDH Analar).

1.4.3. Procedure (modified from Birnboim and Doly, 1979)

1 Pick a single colony of the desired strain of *E. coli* into 5 ml of NB containing selective antibiotics and shake overnight at 37 °C.

2 Subculture 50 µl of this suspension into 500 ml NB containing selective antibiotics in a baffled 2 litre flask and shake overnight at 37 °C.

3 Collect cells by centrifugation in 2 × 250 ml tubes at 8000 rpm and 4 °C for 5 min, using a Sorval RC-5B (or equivalent).

4 Pour off supernatant and resuspend thoroughly in 2 × 20 ml of lysis solution, pool and incubate on ice for 5 min.

5 Add 80 ml of alkaline SDS, mix and incubate for 4 min on ice.

6 Add 60 ml of 3 M KOAc pH 5.2 and mix well.

7 Centrifuge at 8000 rpm and 4 °C for 10 min and pour the supernatant into a clean 250 ml centrifuge tube through a polyallomer wool plug in a plastic funnel.

8 Add 100 ml propan-2-ol, mix well and centrifuge at 8000 rpm and 4 °C for 10 min.

9 Gently rinse the pellet with 70% ethanol and then with ether, and evaporate the ether off in a fume hood.[a]

10 Redissolve the pellet in 10 ml TE.

11 Adjust volume by weighing to 17.5 g (add TE as required), add 20.79 g CsCl and 3.5 ml of EtBr solution. This should give a final density of around 1.55 g/ml.

12 Centrifuge in a 35 ml centrifuge tube at 15 000 rpm at room temperature for 15 min.[b]

13 Completely fill Beckman Ti80 rotor tubes (or equivalent) with the gradient material[c] and heat seal.

14 Centrifuge at 55 000 rpm and 15 °C for 22 h.

15 Remove tubes carefully from the rotor and examine in the dark room under UV light. At this stage two bands should be visible; an upper, sharp band of chromosomal DNA and a lower, diffuse band of plasmid DNA (Fig. 1.10). Puncture the top of the tube and, using a hypodermic syringe and needle inserted through the side of the tube below the lower band, draw off the lower band in as small a volume as possible and transfer it to a sterile Universal bottle.

16 Remove the EtBr completely by partitioning the upper (aqueous) phase several times with an equal volume of propan-2-ol saturated with CsCl and H_2O.

17 Transfer the plasmid solution to a length of prepared dialysis tubing[d] which has been sealed at one end with a Mediclip and seal the other end of the tube in the same way.

18 Dialyse against 2 litres of H_2O for 3 h at 4 °C on a magnetic stirrer, changing the H_2O each hour.

19 Transfer the plasmid solution to a suitable container (e.g. siliconized Corex tube) and determine the plasmid concentration.[e] If the concentration is lower than required, the plasmid will need to be ethanol-precipitated as in steps 20–24.

20 Add 0.1 volumes of 2 M NaOAc pH 5.6 and mix.

21 Add 3 volumes of ethanol, mix and chill in a dry ice/ethanol bath for 15 min.

22 Centrifuge at 10 000 rpm and room temperature for 15 min.

23 Decant the supernatant, rinse the pellet with 70% ethanol and dry in a vacuum desiccator.

24 Redissolve in a small volume of 10 mM Tris–HCl pH 7.5,[f] measure concentration[e] and adjust to 0.5 mg/ml.

Notes

a Ether may be quickly blown off under N_2 using a Pasteur pipette fixed by flexible tubing to a cylinder.

b This spins out all RNA and protein which is precipitated during the preceding step.

c If the tubes cannot be completely filled with the gradient material they must be topped up with a CsCl solution having a density of 1.55 g/ml.

d Dialysis tubing is prepared for use by boiling in 1% Na_2CO_3 for 20 min, soaking in 10 mM EDTA for 20 min at room temperature, washing 2× in dH_2O for 20 min each at room temperature and finally autoclaving in dH_2O.

e DNA concentration may be determined either from comparison with known standards on an agarose gel, or from the OD_{260} of a diluted aliquot of the plasmid solution An OD_{260} of 1 0 = a DNA concentration of 50 μg/ml.

f If plasmid fails to digest with restriction enzymes, then it may be purified by performing a phenol/chloroform extraction between steps 19 and 20 as described below:
(i) Estimate the volume of the plasmid solution, add an equal volume of phenol/chloroform (1 : 1) (see 1.5.2.1) and emulsify by shaking.
(ii) Centrifuge for 5 min at 8000 rpm and remove the upper aqueous phase to a new tube.
(iii) Add an equal volume of chloroform and emulsify by shaking.
(iv) Centrifuge for 5 min at 8000 rpm and remove the upper aqueous phase to a new tube.
(v) Ethanol-precipitate and redissolve as described in steps 20–24.

1.5 PREPARATION AND PURIFICATION OF DNA RESTRICTION FRAGMENTS SUITABLE FOR LIGATION

1.5.1 Background

Details on the use of restriction enzymes are presented in 5.2. Density-gradient purified plasmid is generally very clean and should present no problems for digestion by restriction enzymes. The form in which DNA fragments are ligated will depend upon the strategy adopted for optimizing the recovery of the required recombinant molecule. It may be necessary to isolate specific fragments from the products of a restriction digest and/or dephosphorylate a population of fragments to prevent self-ligation; protocols for these are described below. Before adding to ligation reactions, restriction enzymes must be removed from the DNA solutions by phenol/chloroform extraction and ethanol-precipitation. A discussion of factors to be taken into consideration when attempting to ligate DNA fragments into cloning vectors is presented in 1.6. For our example pBin19 will be cut with *Hind* III and dephosphorylated, whilst pUC18CaMVCAT will be cut with *Hind* III and the 4.6 kb CaMV-*cat* fragment of pUC18CaMVCAT purified via electro-elution. Lanes 2, 4 and 5 of Fig. 1.10 show which DNA fragments are present during different stages of the fragment preparation.

1.5.2. Purification of DNA fragments from a restriction digest

1.5.2.1 Materials

Equipment/consumables

As in 5.2.

Solutions

As in 5.2, plus:

- Phenol/chloroform (1 : 1). Mix in a 1 : 1 ratio TE-saturated phenol (see 1.4.2 for TE) containing 0.1% 8-hydroxyquinoline and chloroform containing 4%

isoamyl alcohol. This is stable for several months at 4 °C and should not be used if the normally bright yellow colour becomes tinged with brown.

- 2 M NaOAc pH 5.6 (1.4.2).
- 70% ethanol.
- Chloroform (BDH Analar).

1.5.2.2 Procedure

Perform a restriction digest on appropriate plasmid as outlined in 5.2.

1. After the digestion has run to completion, make up volume to 100 µl with H_2O.
2. Add 100 µl phenol/chloroform (1 : 1) and emulsify by shaking vigorously.
3. Centrifuge in a minifuge (at 12 000 rpm) for 5 min and remove upper aqueous phase to a new Eppendorf tube.
4. Add 100 µl chloroform, mix well and centrifuge at 12 000 rpm for 1 min.
5. Using a Gilson, remove the lower organic phase and discard.
6. Add 10 µl 2 M NaOAc pH 5.6 and mix.
7. Add 330 µl ethanol and chill in a dry ice/ethanol bath for 15 min.
8. Centrifuge at 12 000 rpm for 15 min and decant the supernatant.
9. Add 330 µl 70% ethanol, mix and recentrifuge at 12 000 rpm for 5 min.
10. Decant the supernatant, vacuum-dry and resuspend in an appropriate volume of H_2O for adding to a ligation reaction (1.6).

1.5.3 Removal of 5′ phosphate groups from DNA fragments using calf intestinal phosphatase

1.5.3.1 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).

Solutions

- 10× CIP (500 mM Tris-HCl pH 8.0, 1.0 mM EDTA). *For 10 ml:*

2 M Tris-HCl pH 8.0 (see 1.4.2)	2.50 ml
0.5 M EDTA	0.02 ml.

Make up to volume with dH_2O.

Cont

- 30 mM Na nitrilotriacetic acid. *For 10 ml:* dissolve 70.5 mg of disodium salt (Sigma) in 10 ml of dH_2O. Store at −20 °C.
- Calf intestinal phosphatase (BCL grade for molecular biology) diluted to required activity[a] in storage buffer.

1.5.3.2 Procedure

1 Add the following components to a 1.5 ml Eppendorf tube:

DNA solution (in H_2O)	1–17 µl
10× CIP	2 µl
H_2O to 19 µl	
Calf intestinal phosphatase	1 µl.

2 Incubate at 37 °C for 30 min.

3 Stop reaction by adding 10 µl 30 mM nitrilotriacetic acid and incubating at 65 °C for 45 min.

If the phosphatased DNA is to be added directly to a ligation reaction it should be purified as described in 1.5.2. If specific phosphatased fragments are to be isolated, the stopped reaction mixture should be treated in the same way as a completed restriction digest in either of the two protocols in 1.5.4 or 1.5.5.

Note

a 0.05 U enzyme activity are required to remove the 5′ terminal phosphate groups from 1 pmol of ends (1.6 µg of a 5 kb fragment contains 1 pmol ends). It is important that the minimum amount of enzyme is used as single-stranded sticky ends may be digested away by the low levels of exonucleases which seem to contaminate preparations of this enzyme.

1.5.4 Electroelution of restriction fragments from agarose gels

1.5.4.1 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).
- Equipment for agarose gel electrophoresis (5.3.2).
- Electroelution tank (e.g. LKB; Extraphor).
- UV transilluminator (e.g. UV Products; model TS-36).
- UV safety goggles and full face visor.
- Blunt-ended forceps.

Solutions

- Solutions for agarose gel electrophoresis (5.3).

- High-salt buffer (3 M NaOAc pH 7.9, 0.01% bromophenol blue). *For 100 ml:* dissolve 40.82 g of sodium acetate trihydrate (BDH Analar) in 50 ml of dH_2O. Titrate to pH 7.9 with glacial acetic acid (BDH Analar), add 10 mg of bromophenol blue (Pharmacia) and make to 100 ml. Autoclave and store at room temperature.
- Running buffer: (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM NaCl). *For 1 litre of a 10× stock:*

2 M Tris-HCl pH 8.0 (see 1.4.2)	50 ml
0.5 M EDTA	20 ml
5 M NaCl	1 ml
dH_2O	929 ml.

This should be stored as a 10× stock at room temperature and diluted to working strength (1×) just before use.

- Ethanol (−20 °C).
- 70% ethanol.
- Sterile dH_2O.

1.5.4.2 Procedure

1 Electrophorese restricted DNA on an agarose gel as described in 5.3. The amount of digested DNA and gel electrophoresis conditions should be adjusted to give good separation of the desired fragment from other DNA and to produce sufficient DNA in the band to be excised for subsequent ligation (1.6.1.).

2 Place gel on a UV transilluminator and identify which bands are required.

3 Using a new scalpel blade, cut around the required bands (being careful not to damage the UV filter) and transfer to sterile plastic Universals with fine forceps.

4 Set up the electroelution tank as described by manufacturers.

5 Cut the gel fragment(s) into small pieces (1–3 mm cubes) and place into the circular wells of the electroelutor.

6 Electrophorese at 150 V for 45 min, after which the DNA should be trapped in the high-salt buffer.

7 Using a 100 µl micropipette transfer the high-salt buffer in a volume of 300 µl to an Eppendorf tube.

8 Add 1 ml of cold (−20 °C) ethanol and chill in a dry ice/ethanol bath for 15 min.

9 Centrifuge at 12 000 rpm for 15 min and decant the supernatant.

Cont.

10 Add 500 μl 70% ethanol, mix, recentrifuge at 12 000 rpm for 5 min, decant the supernatant and vacuum-dry.

11 Redissolve in an appropriate volume of H_2O for adding to a ligation (1.6).

1.5.5 Purification of specific restriction fragments by centrifugal filtration

1.5.5.1 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1[I]).
- Equipment for agarose gel electrophoresis (5.3.2).
- UV transilluminator (e.g. UV Products Ltd; model TS-36).
- UV safety goggles and full face visor.
- Blunt-ended forceps.
- 2.5 cm × 2.5 cm pieces of Millipore GVWPO4700 filter or Genescreen (BRL (UK) Ltd.).
- Plastic stirring rods (W. Sarstedt & Co.).

Solutions

- Solutions for agarose gel electrophoresis (5.3).
- Elution buffer (50 mM Tris-HCl pH 7.5, 0.1% SDS). *For 100 ml:*
  2 M Tris-HCl pH 7.5 (1.4.2) 2.5 ml
  25% SDS 0.4 ml.
  Make up by adding components to 97.9 ml H_2O.
- Phenol/chloroform (1 : 1) (1.5.2.1).
- 5 M NaCl.
- 70% ethanol.
- Sterile dH_2O.

1.5.5.2 Procedure (modified from Zhu *et al.*, 1985)

1 Cut out a restriction fragment from an 0.8% agarose gel in as small a volume as possible as outlined in 1.5.4.2.

2 Assemble the filter unit as follows:
(a) Carefully cut the lower conical section from a 1.5 ml Eppendorf tube and pierce a hole in the bottom using a fine syringe needle.
(b) Wet a 2.5 cm square piece of Millipore GVWPO4700 filter or Genescreen with elution buffer, fold into quarters and, using a thin, round-ended stirring rod and taking care not to rip the filter, push into the conical filter-holder.

3 Place the gel fragment into the filter unit, sit the filter unit in a 1.5 ml Eppendorf tube from which the cap has been removed and centrifuge at 12 000 rpm for 10 min and then remove filter unit containing agarose.

4 Add 100 μl phenol/chloroform (1 : 1) to the filtrate, emulsify by shaking vigorously, and centrifuge at 12 000 rpm for 5 min.

5 Remove upper aqueous phase to a new tube.

6 Repeat steps 4 and 5.

7 Add 100 μl chloroform, mix and centrifuge at 12 000 rpm for 1 min.

8 Using a Gilson, remove the lower organic phase and discard.

9 Estimate the volume of the DNA solution and add 0.25 volumes of 5 M NaCl.

10 Add 3 volumes of ethanol and chill in a dry ice/ethanol bath for 15 min.

11 Centrifuge at 12 000 rpm for 15 min and decant supernatant.

12 Add 500 μl 70% ethanol, recentrifuge at 12 000 rpm for 5 min and decant supernatant.

13 Vacuum-dry pellet and then redissolve in an appropriate volume of H_2O to add to a ligation reaction (1.6).

1.6 LIGATION OF DNA FRAGMENTS INTO PLANT TRANSFORMATION VECTORS

1.6.1. Background

Ligation is an energy-dependent reaction in which fragments of double-stranded DNA, containing blunt or sticky ends (i.e. 5′ or 3′ protruding ends), are covalently joined. 5′ phosphate groups are condensed with closely associated 3′ hydroxyl groups to form a continuous sugar–phosphate backbone.

When considering the form of ligation products, two factors, i and j, are important.

i is the total concentration of self-complementary ends in the reaction and is a measure of the degree to which an end can interact with another complementary end on a different molecule.

When a fragment has similar, complementary ends then:

$$i = 2N_0\text{M} \times 10^{-3} = 1.2 \times 10^{21}\text{M ends/ml.}$$

When a fragment has different, non-complementary ends, then for each type of end:

$$i = N_0\text{M} \times 10^{-3} = 6.0 \times 10^{20}\text{M ends/ml,}$$

where N_0 is Avogadro's number and M is the molar concentration of DNA molecules.

j is the effective concentration of one end in the vicinity of the other end of the same molecule and is a measure of the degree to which an end can interact with the other complementary end of the same molecule. The value of j is determined by the assumption that the DNA exists as a random coil:

$$j = \left[\frac{3}{2\pi lb}\right]^{2/3} = j\lambda\left[\frac{MW\lambda}{MW}\right]^{3/2} = \frac{6.2 \times 10^{22}}{(MW)^{3/2}} \text{ ends/ml},$$

where l is the contour length, b is the random coil segment length and MW is the molecular weight of a DNA molecule, and $j\lambda$ and $MW\lambda$ are the respective values for bacteriophage λ.

When considering a population of uniformly long DNA fragments, each with two complementary ends, the form of ligation products can be predicted from the ratio $j:i$. When $j:i = 1$, equal proportions of circular monomers and linear dimers are predicted. $j:i > 1$ will tend towards the formation of circular monomers and $j:i < 1$ towards linear concatamers. A rearrangement of the above equations allows the DNA concentration in moles/l (M) required to achieve a certain $j:i$ ratio to be calculated:

$$[\text{DNA}] = \frac{i}{j} \times \frac{51.1}{MW^{3/2}} \text{ M} \quad \text{when } i = 2N_0M \times 10^{-3} \text{ ends/ml}$$

or

$$[\text{DNA}] = \frac{i}{j} \times \frac{25.6}{MW^{3/2}} \text{ M} \quad \text{when } i = N_0M \times 10^{-3} \text{ ends/ml}$$

In practice, the situation is more complex than that described above. The fragments used when cloning into plasmids are rather short for the random coil model to predict their behaviour accurately and it is generally found that linear concatomers predominate when $j:i = 1$. Ligations are usually performed between molecules of different length and hence different j values, which therefore require different concentrations of ends to form the same kinds of products. When cloning into plasmids the required end-product is generally a circular heterodimer which necessitates a compromise between a low $j:i$ ratio to promote initial intermolecular ligation and a high $j:i$ ratio to promote subsequent recircularization. Additionally, the $j:i$ ratio will change during a reaction, i decreasing as ends become ligated and hence unavailable as substrates, and j decreasing as molecules become longer. As a ligation proceeds $j:i$ becomes larger and circularization is favoured.

Empirically, the optimal $j:i$ ratio for the recovery of circular heterodimers is about 2, and this should generally be calculated for the smaller of the two molecules. *NB: do not forget that i is the total concentration of each type of end in the reaction, and thus is contributed to by both types of fragment present.* Since the desired product contains one vector molecule and one insert, they should both be present in the same molar concentration.

Various strategies are used to increase the yield of the circular heterodimer, one of which, dephosphorylation, is described in 1.5.3. These methods generally

Table 1.3 Properties of a vector plasmid and an insert

Fragment	Size (kb)	Molecular weight	j (ends/ml)
CaMV-*cat* × *Hind* III	4.6	3.1×10^6	1.1×10^{13}
pBin19 × *Hind* III	10.0	6.7×10^6	3.6×10^{12}

prevent recircularization of one or both of the fragments until an intermolecular ligation has taken place. Unless one set of fragments contains unmodified self-complementary ends, these strategies allow ligations to be performed at higher $j : i$ values to enhance recircularization of recombinant molecules.

The use of these equations is illustrated by the following example in which the 4.6 kb CaMV-*cat* fragment from a *Hind* III digest of pUC18CaMVCAT is ligated into the dephosphorylated *Hind* III site of pBin19 to form pBin19CaMV-*cat*. Restriction maps of the two plasmids and the cloning strategy are given in Fig. 1.7. The relevant properties of the vector plasmid and insert are presented in Table 1.3.

It is important for the unmodified insert to be present in the ligation at the correct $j : i$ ratio in order to achieve maximal yields of the desired circular heterodimer. Since the vector has been modified by alkaline phosphatase it may only be ligated to the unmodified insert ends, and therefore the $j : i$ ratio for the vector is unimportant. However, the desired product of the reaction contains one vector molecule and one insert molecule; hence they should be present in equimolar amounts in the ligation reaction:

$$[\text{DNA}] \text{ to obtain } j : i = 2 \text{ for insert} = \frac{1}{2} \times \frac{51.1}{(3.1 \times 10^6)^{3/2}} \text{ M.}$$

$$= 4.7 \times 10^{-9} \text{ M}$$

Since the molecular ratio of vector to insert required is 1 : 1, each fragment must be present at

$$\frac{4.7 \times 10^{-9}}{2} \text{ M} = 2.3 \times 10^{-9} \text{ M.}$$

Therefore the 20 μl of ligation reaction will contain:

$4.7 \times 10^{-9} \times 3.1 \times 10^6 \times 20\,\mu g = 291$ ng insert (CaMV-*cat* × *Hind* III),
$4.7 \times 10^{-9} \times 6.7 \times 10^6 \times 20\,\mu g = 630$ ng vector (pBin19 × *Hind* III).

Similar calculations can be used to ascertain ligation conditions for the cloning of the same fragment into a small intermediate vector before co-integration into *vir* helper plasmids such as pGV3850 at a later stage.

Not all the resultant recombinant DNA molecules obtained from the ligation process will be those that are required. Ligations result in a variety of molecules which can include multimers of the insert or vector, self-ligated vector sequences and deleted molecules arising from the action of nucleases on the DNA. Following

transformation of *E coli* with the ligated molecules (1.7) it is necessary to identify colonies containing the desired recombinant DNA molecules. This is generally a two-step process, requiring first identification of the bacterial colonies harbouring insert-containing plasmids (this can involve several techniques such as dominant marker inactivation, expression of new phenotypic characters, or hybridization of the DNA from lysed bacterial colonies to radioactive probes), and second, the isolation of the cloned DNA by a plasmid 'miniprep' (1.8) followed by restriction enzyme digestion and analysis on an agarose gel (5.3).

Blunt-end ligation is less efficient than sticky-end ligation and is performed under different incubation conditions. A protocol for the sticky-end reaction is described below using pBin19 as an example vector and the *Hind* III fragment CaMV-*cat* as an example insert. A protocol for blunt-end ligation is also presented.

1.6.2 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).
- 4 °C ('sticky-end' ligation) or 22 °C ('blunt-end' ligation) water bath.

Solutions

- Plasmid DNA solutions: e.g. pBin19 plasmid (125 ng/μl) and dephosphorylated, pBin19 cut with *Hind* III (125 ng/μl), *Hind* III CaMV-*cat* fragment (60 ng/μl).
- 10× ligation buffer (660 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol, 10 mM ATP). *For 1 ml:*

2 M Tris-HCl pH 7.6	330.0 μl
$MgCl_2$ (Sigma, hexahydrate)	10.2 mg
1 M DTT (Sigma)	50.0 μl
ATP (Pharmacia, disodium salt)	6.1 mg
dH_2O	to 1.0 ml.

This should be divided into 10 μl aliquots, stored at −20 °C, thawed on ice just before use and used once only.

- 100 mM Spermidine. *For 1 ml:*

Spermidine (Sigma, trihydrochloride)	25.5 mg
dH_2O	to 1.0 ml.

- 20 mM ATP. *For 1 ml:*

ATP (Pharmacia, disodium salt)	12.2 mg
dH_2O	to 1.0 ml.

- T_4 DNA ligase (New England Biolabs 400 000 U/ml) diluted to 0.1 U/μl (sticky-end ligation) or 1.0 U/μl (blunt-end ligation) with the storage buffer recommended by the manufacturers.

Table 1.4 Series of control reactions to be performed with the ligation example using pBin19 as a vector and *Hind* III fragment CaMV-*cat* as an insert

	Tube number					
Additions (μl)	1	2	3	4	5	6
Uncut vector	10	10	—	—	—	—
Cut vector	—	—	10	10	—	—
Cut and dephos. vector	—	—	—	—	10	5
Cut insert	—	—	—	—	—	5
10× ligation buffer	—	2	2	2	2	2
100 mM spermidine	—	1	1	1	1	1
20 mM ATP	—	1	1	1	1	1
dH_2O	10	5	6	5	5	5
T_4 DNA ligase	—	1	—	1	1	1
Total volume (μl)	20	20	20	20	20	20

1.6.3 Procedure (modified from Maniatis *et al.*, 1982)

The procedure below is a general protocol for the insertion of DNA restriction fragments into small cloning vectors. However, besides calculating the optimum amounts of both vector and insert DNA to include in the ligation reaction as outlined above, many factors can affect the efficiency of both the ligation and the subsequent transformation steps following the incubation of the ligation mixtures with competent *E. coli* cells (1.7). Thus, it is common practice to carry out a series of control reactions to pinpoint the problem areas in case the results are poor. The series of controls which should be performed with the ligation example described above are presented in Table 1.4. Typical results showing the number of transformants recovered following the transformation of these ligation mixtures into *E. coli* strain JM83 are presented in Table 1.5.

Explanation. The results of a ligation are generally seen only after transformation of competent cells. The set of controls outlined above is designed to demonstrate that all the steps leading up to and including transformation produce the expected results in terms of the proportion of transformable molecules, so that departures from the expected frequencies in any tube should pinpoint the stages at which problems are occurring.

Tube 1 This is a transformation control to assess transformation efficiency of cells using the uncut vector.

Tube 2 This demonstrates the effect of any of the non-DNA reaction components on transformation efficiency when compared with the number of colonies obtained from tube 1.

Tube 3 This control ascertains the background rate of transformation from restricted vector DNA.

Tube 4 When compared to the number of colonies produced from tube 3 this demonstrates that the presence of ligase is resulting in an increase of transformable molecules.

Tube 5 When compared with the numbers of colonies produced from tubes 3 and 4, this demonstrates that dephosphorylation both maintains transformation at similar frequencies to those achieved with linear vector and prevents the increase in transformation demonstrated for unmodified vector in the presence of ligase.

Tube 6 This is the experimental tube.

Sticky-end ligation

1 Transfer vector DNA and insert DNA into Eppendorf microfuge tubes in a total volume of up to 15 μl and place on ice.

2 Add in the following order:

10× ligation buffer[a]	2 μl
100 mM spermidine	1 μl
20 mM ATP	1 μl
dH_2O to 19 μl	
0.1 U/μl T_4 ligase	1 μl.

3 Mix and incubate at 4 °C overnight.[b]

Blunt-end ligation

Blunt-end ligations are performed in essentially the same way as a sticky end reaction except that a greater amount of ligase is used and the incubation temperature is 22 °C, rather than 4 °C.

1 Transfer vector DNA and insert DNA into Eppendorf microfuge tubes in a total volume of up to 15 μl and place on ice.

2 Add in the following order:

10× ligation buffer[a]	2 μl
100 mM spermidine	1 μl
20 mM ATP	1 μl
H_2O to 19 μl	
1.0 U/μl T_4 ligase	1 μl.

3 Mix and incubate at 22°C overnight.[b]

Notes

a Ligation buffer must be thawed once on ice and used immediately.

b Approximately 10 ng of DNA from each ligation reaction should be added to competent cells for transformation. For the presented example, the ligation should be made up to 185 μl with dH_2O and 2 μl added directly to competent cells as described in 1.7.3.

1.7 INTRODUCTION OF RECOMBINANT PLASMIDS INTO *E. COLI* VIA TRANSFORMATION

1.7.1 Background

Transformation of *E. coli* by naked DNA was first demonstrated by Mandel and Higa in 1970, and was achieved by incubating bacteria with bacteriophage λ DNA in a solution of $CaCl_2$ at 0 °C. Cohen *et al.* (1972) effected transformation of *E coli* with plasmid using essentially the same conditions. Subsequent development of plasmid transformation protocols has progressed by empirical means and modern procedures involve the production of 'competent' cells (i.e. competent for transformation) which can either be used fresh or stored frozen, still in a competent state, and then used at a later date. Most methods incorporate an interaction of bacteria and plasmid at low temperature in the presence of divalent cations, but several additional factors have been shown to enhance the reliability and efficiency of transformation. These include the strain and growth conditions of the bacteria used, the quality of the water and chemicals used for making up solutions, the cleanliness of glassware and the addition of dimethylsulphoxide and dithiothreitol to transformation buffers (see discussion in Glover, 1985).[a]

When attempting to transform *E. coli* with recombinant plasmids from ligation reactions it is important to perform transformation controls both to assess the transformation efficiency of the competent cells used and to check the stringency of the selection and screening procedures. As indicated previously in 1.6, when using ligation mixtures rather than intact plasmids for transformation, other types of controls are normally performed to monitor both the efficiency of the ligation in producing circular heterodimers, and the amount of self-ligation occurring (Table 1.4). The results from a typical ligation and transformation experiment are presented in Table 1.5.

A relatively simple protocol is described below which has been optimized[a] for *E. coli* strain JM83[b] and should yield transformation frequencies in the range of $1\text{–}5 \times 10^7$ transformants/μg[c] DNA from cells which have been stored at −80 °C in a competent state.

In the example given, colonies containing the desired recombinant plasmid grow and appear white on NA + Km + X-GAL + IPTG plates. Km resistance is due to the presence of pBin19 sequences and the white colour is a consequence of the insertional inactivation of the β-galactosidase gene of pBin19 by the CaMV-*cat* fragment. JM83 is *Lac*$^-$ and therefore colonies harbouring plasmids with a functional β-galactosidase gene (i.e. pBin19 lacking inserts) are able to cleave X-GAL and appear blue (Gronenborn and Messing, 1978).

When performing a transformation it is common practice to include a transformation control in which transformation efficiency is determined by transforming an aliquot of competent cells with a known, non-saturating amount of an uncut plasmid (e.g. 1 ng pBR322). It is a good idea to use a plasmid for the transformation control which is similar in size to the expected recombinant plasmid in

order to avoid underestimation of ligation efficiency.[d] In the example presented, the transformation control is tube 1 of the ligation reactions in Table 1.4.

1.7.2 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).
- 37 °C environmentally controlled orbital shaker with clips for Universal bottles and 1 litre conical flasks.
- 37 °C water bath.
- 37 °C static incubator.
- 35 ml polyallomer centrifuge tubes with polypropylene screw caps (e.g. Nalgene type 3119).
- Refrigerated, medium-speed centrifuge (e.g. Sorval RC-5B or RT600B).
- Spectrophotometer to read OD_{550} (e.g. Perkin–Elmer).
- Disposable 1 ml cuvettes (Sarstedt).
- Small Dewar flask containing liquid nitrogen.
- −80 °C freezer.

Bacteria

- Selection plates containing colonies of transformation host bacteria e.g. JM83 on NA + 12.5 μg/ml Sm (1.3.4).

Solutions[e]

- Ligation reactions (1.6).
- Control uncut plasmid DNA (e.g. pBin19 at 125 ng/μl).
- ψ-broth (2% [w/v] Difco Bacto Tryptone, 0.5% [w/v] Difco Bacto Yeast Extract, 0.4% [w/v] $MgSO_4$, 10 mM KCl. *For 1 litre*

Difco Bacto Tryptone	20.00 g
Difco Bacto Yeast Extract	5.00 g
$MgSO_4 \cdot 7H_2O$ (BDH Analar)	4.00 g
KCl (BDH Analar, anhydrous)	0.75 g.

Sterilize by autoclaving and dispense; 5 ml in sterile plastic Universal bottles and 100 ml in 1 litre conical flasks.

- TFB 1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% glycerol). *For 50 ml*

1 M RbCl (Sigma)	5.00 ml
$MnCl_2 \cdot 4H_2O$ (Sigma, tetrahydrate)	0.495 g
KOAc (BDH Analar)	0.147 g
750 mM $CaCl_2 \cdot 2H_2O$ (Sigma, dihydrate)	0.67 ml
50% glycerol (BDH Analar)	15.00 ml.

Adjust pH to 5.8 with glacial acetic acid, make up to volume with H_2O and filter-sterilize.

- TFB 2 (10 mM MOPS pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol). *For 50 ml:*

100 mM MOPS (Sigma) pH 7.0 (with NaOH)	5.0 ml
1 M RbCl (Sigma)	0.5 ml
750 mM $CaCl_2$ (Sigma, dihydrate)	5.0 ml
50% glycerol (BDH Analar)	15.0 ml.

Make up to volume with dH_2O and filter-sterilize.

- Bacterial selection plates. In this example the selection plates quoted are those used for the plant transformation vector pBin19:
  NA
  NA + Km
  NA + Km + X-GAL + IPTG.

Different antibiotics may be necessary for other vectors and the use of the X-GAL/IPTG indicator system may not be appropriate.

1.7.3 Procedure

Production of competent E. coli cells

1 Using a sterile loop pick off[f] a single colony from a freshly streaked bacterial plate (e.g. JM83), resuspend in 5 ml ψ-broth in a sterile Universal and shake at 37 °C for 2–3 h.

2 Inoculate this starter culture into 100 ml prewarmed ψ-broth in a 1 litre conical flask, and shake at 37 °C until OD_{550}[g] reaches 0.35 (i.e. the culture is in the logarithmic phase of growth and contains $3.5 - 4.0 \times 10^7$ cells/ml). This generally takes about 2–3 h. OD_{550} is determined on 1 ml samples of the culture removed at frequent intervals with a sterile Pasteur pipette.

3 Transfer the culture to 2 × 35 ml capped centrifuge tubes and chill on ice for 15 min.[h]

4 Centrifuge at 2500 rpm for 5 min at 4 °C and decant the supernatant.

5 Resuspend the cells by gentle vortexing in 10.5 ml ice-cold TFB 1, pool and incubate on ice for 90 min.

6 Centrifuge at 2500 rpm for 5 min at 4 °C and decant the supernatant.

7 Resuspend the cells gently in 2.8 ml ice-cold TFB 2 and aliquot 200 μl into 1.5 ml Eppendorf microfuge tubes. These cells are now competent and may either be used immediately for transformation or flash-frozen in liquid nitrogen and stored for many months at −80 °C with little reduction in transformation efficiency.

Transformation of competent E. coli cells

1 Thaw frozen, competent cells at room temperature until just molten and incubate on ice for 10 min. Alternatively, use freshly prepared competent cells.

2 Add up to 10 ng DNA in a maximum volume of 10 μl of H_2O (e.g. 2 μl of 10-fold diluted ligation mixtures from 1.6; Table 1.2), mix gently, and incubate on ice for 20 min.

3 Heat shock the cells by placing the tubes in a 37 °C water bath for 60 sec and return immediately to ice for a further 2 min.

4 Add 800 μl ψ-broth and shake at 37 °C for 50 min.[1]

5 Spin the cells for 30 sec in a microfuge, pour off the supernatant and resuspend the cells in 100 μl ψ-broth.

6 Make serial dilutions to 10^{-6} of the cell suspensions in 10-fold steps by adding 10 μl cell suspension to 90 μl ψ-broth. Using a flame-sterilized and cooled glass spreader, spread the tube contents of the 10^{-6} and 10^{-5} dilutions onto NA plates (these are cell viability controls), and those of the 10^{-2}, 10^{-1} and 10^{0} dilutions onto NA plates containing appropriate selective antibiotics (e.g. Km in the case of pBin19) and onto plates containing both antibiotics and X-GAL + IPTG when using vectors incorporating a *lacZ* screening system for recombinant transformants (Table 1.5).

7 Allow the plates to dry in the stream of sterile air generated by a flow hood.[1]

8 Incubate upside-down overnight at 37 °C.

9 Estimate the total number of colonies on plates by choosing a fraction of a plate which contains a managable number of colonies (e.g. 50–500), counting the colonies in that fraction and multiplying by the reciprocal of that fraction. Calculate the transformation frequency on the transformation control plates in terms of number of transformants/μg DNA and compare with the expected value to verify that the transformation has worked. The results of a typical ligation/transformation experiment in which the 4.6 kb *Hind* III fragment of pUC18CaMVCAT is inserted into *Hind* III cut and dephosphorylated pBin19 to form pBin19CaMV-*cat* (1.6.3) are given in Table 1.5.

The cell viability:

$$\frac{186 \times 10^6}{5} = 3.7 \times 10^7 \text{ cells/ml.}$$

The transformation frequency for pBin19:

$$698 \times 10^2 \times \frac{1000}{12.5} = 5.6 \times 10^6 \text{ transformants/}\mu\text{g.}$$

Table 1.5 Results from a typical ligation and transformation experiment

Tube	Plate	Dilution	No. of Colonies	
			Blue	*White*
1	NA	10^{-6}	185	
	NA + Km	10^{-2}	698	
	NA + Km	10^{-3}	73	
2	NA + Km	10^{-2}	659	
	NA + Km	10^{-3}	59	
3	NA + Km	10^{0}	52	
4	NA + Km	10^{-2}	272	
	NA + Km	10^{-3}	25	
5	NA + Km	10^{-0}	69	
6	NA + Km + X-GAL + IPTG	10^{0}	34	86
	NA + Km + X-GAL + IPTG	10^{-1}	2	9

The percentage inhibition of transformation by other components of the ligation mixture:

$$\frac{698 - 659}{698} \times 100 = 6\%.$$

The efficiency of transformation by restricted pBin19:

$$\frac{52}{659 \times 10^2} \times 100 = 0.08\%.$$

The efficiency of self-ligation of pBin19:

$$\frac{272 \times 10^2}{659 \times 10^2} \times 100 = 41\%.$$

The inhibition of self-ligation by dephosphorylation:

$$\frac{272 \times 10^2 - 69}{272 \times 10^2} \times 100 = 99\%.$$

Total number of recombinant colonies/μg of ligated DNA:

$$86 \times \frac{925}{10} \times \frac{1000}{925} = 8600 \text{ colonies}/\mu\text{g}.$$

Notes

a During optimization of the transformation protocol, the following usually have the greatest effects on transformation efficiency:
(i) The OD_{550} at which the cells were harvested.
(ii) Flash-freezing competent cells in liquid nitrogen.
(iii) Adding DNA in as small a volume of H_2O as possible.
(iv) Heat-shocking at 37 °C rather than the more traditional 42 °C.
If this protocol is to be optimized for a strain other than JM83, or transformation efficiencies are not up to the levels quoted, then investigating the above conditions may prove beneficial.

b JM83 has proved to be a suitable strain for transformation work in many laboratories for the following reasons:
(i) It exhibits usefully high transformation frequencies.
(ii) It gives consistently high yields of plasmid from both large-scale and rapid plasmid isolation procedures.
(iii) It contains a chromosomal Sm-resistance gene which allows it to be selected from contaminating bacteria.
(iv) It is *recA*$^-$ which allows efficient and reliable cloning of recombinant DNA.
(v) It is *lac* which allows phenotypic screening for the presence of inserts when cloning into plasmids containing appropriate *LacZ* constructs, such as those in pBin19 and pUC18.

c The maximum number of transformants obtainable from a batch of competent cells is dependent on two factors; the proportion of competent cells in the batch and the transformation efficiency of those competent cells. The proportion of competent cells can be determined by comparing the number of transformants obtained from a saturating amount of plasmid (e.g. 500 ng) to the total number of viable cells in the aliquot used for transformation, and for the protocol described above should be between 1.5 and 2.0%. The transformation efficiency can be determined from the number of transformants obtained from a non-saturating amount of DNA (e.g. 50 pg), and should be 10^7 – 10^8 transformants/μg DNA with the above protocol.

d Since transformation efficiency is expressed on a per weight basis rather than on a per molecule basis, apparent transformation efficiency decreases linearly with increasing plasmid size.

e Transformation is very sensitive to the purity of chemicals and water used in solutions — always use the purest available.

f Do not transfer any agar as it is inhibitory to transformation.

g Viable cell density is proportional to OD_{550} over a wide range, but the exact relationship is strain-dependent.

h It is very important that, once the cells have been chilled, they are kept on ice as much as possible and are only removed for operations such as mixing, which should be performed quickly and gently.

i This is to allow the expression of antibiotic-resistance genes which have been introduced via transformation.

j Wet agar is inhibitory to colony formation during incubation.

1.8 RAPID SMALL-SCALE PREPARATION OF *E. COLI* PLASMID DNA

1.8.1 Background

The plasmid miniprep is based on the same principles as those described for the large-scale plasmid isolation. Variations of the following protocol have been used successfully in many laboratories with a range of plasmid/bacterial strain combinations. The density gradient purification is omitted to save time and hence the plasmid DNA is not normally clean enough to be used for any purpose other than to check for the correct construction of plasmids in transformed bacteria by restriction analysis (1.9), and Southern blotting if necessary. In the case of plant transformation vectors, it is essential to check the structure of recombinant plasmids in *E. coli* before attempting transfer to *Agrobacterium* by conjugation (1.10). A restriction analysis (Fig. 1.11) of plasmid minipreps derived from randomly picked transformed colonies of JM83 thought to contain recombinant pBin19 plasmids (i.e. white, Km^r colonies from NA + Km + X-GAL + IPTG plates) is represented in 1.9.

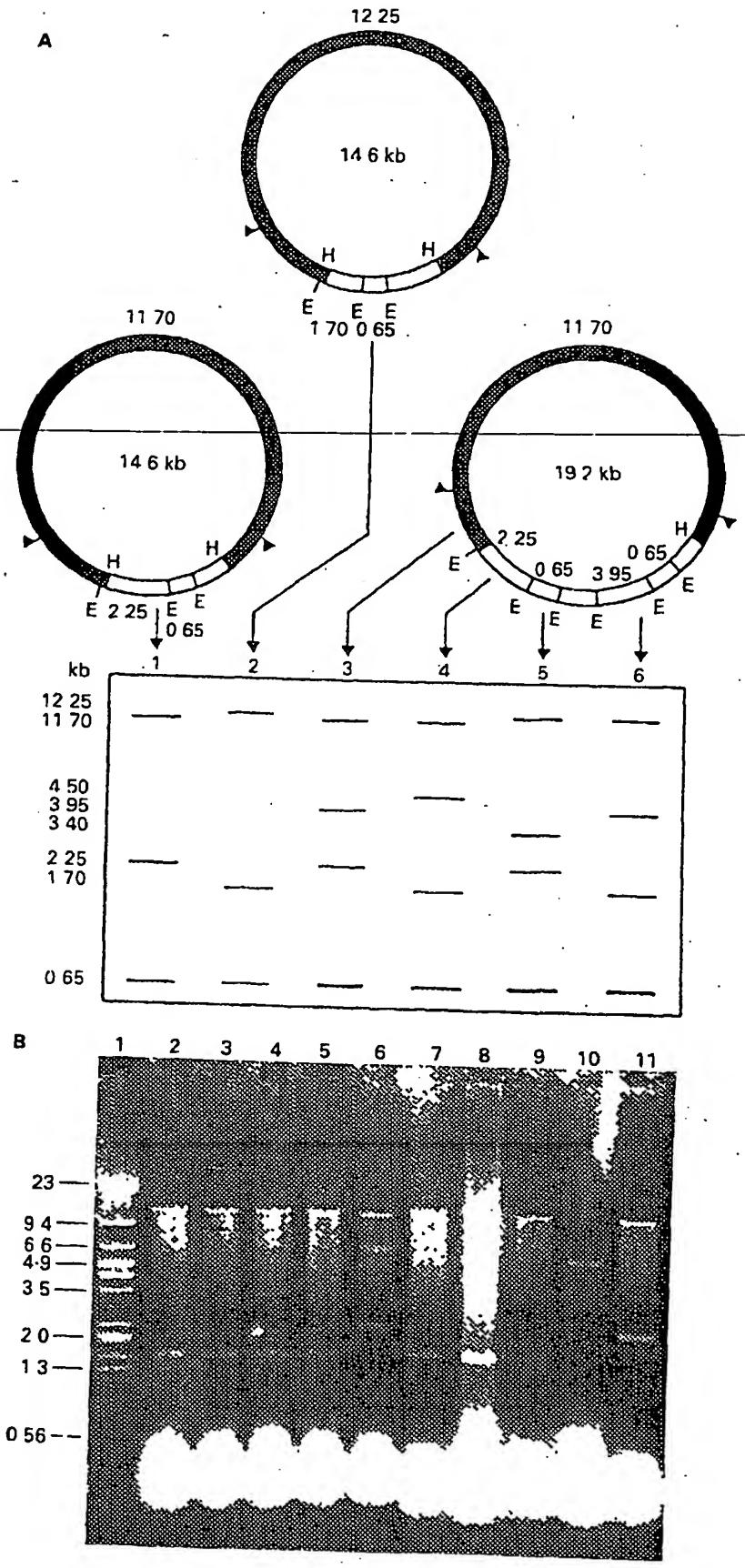
1.8.2 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).
- 37 °C orbital shaker with clips for Universal bottles (e.g. Gallenkamp).
- Dry ice/ethanol bath.

Solutions

- NB + selective antibiotics for the strain(s) of bacteria used (5 ml in Universals).
- Lysis solution: see 1.4.2.
- Alkaline SDS: see 1.4.2.
- 3 M NaOAc pH 4.8. *For 400 ml:* Dissolve 98.44 g of NaOAc (BDH Analar, anhydrous) in 200 ml H_2O. Titrate to pH 4.8 with glacial acetic acid (BDH Analar), make to 400 ml, autoclave and store at room temperature.
- TE: see 1.4.2.
- 2 M NaOAc pH 5.6: see 1.4.2.
- Phenol/chloroform (1 : 1): see 1.5.2.1.
- Chloroform.
- Ethanol (−20 °C).


A
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1 70 0 65
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1 70
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9 4
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1 3
0 56

1.8.3 Procedure (modified from Birnboim and Doly, 1979)

1 Pick single colonies of clones harbouring putative recombinant plasmids into 5 ml NB containing selective antibiotics (e.g. 50 μg/ml Km for pBin19CaMV-*cat* in *E coli*), and shake overnight[a] at 37 °C.

2 Pour 1.5 ml of cells into a microfuge tube and collect the cells by centrifugation at 12 000 rpm for 2 min.[b]

3 Remove all of the supernatant and resuspend cells in 100 μl of lysis solution. Stand on ice for 5 min.

4 Add 200 μl of alkaline SDS and gently mix until the suspension is clear and viscous. Leave on ice for 5 min.

5 Add 150 μl of 3 M NaOAc (pH 4.8) and invert tube several times while the DNA clot is forming. Leave on ice for 10 min.

6 Centrifuge in microfuge for 10 min (this should yield a clear supernatant) and remove the supernatant to a new tube.

7 Add 1 ml of cold (−20 °C) ethanol to supernatant, mix well and leave in a dry ice/ethanol bath for 15 min.

8 Collect the precipitate by centrifugation at 12 000 rpm (full speed in a microfuge) for 10 min.

9 Gently pour off supernatant and dissolve the pellet in 100 μl of TE.

10 Add 100 μl phenol/chloroform, mix well and separate the phenolic and aqueous phases by centrifugation at 12 000 rpm for 10 min.

11 Remove the upper aqueous phase to a new tube, add 100 μl chloroform, mix and separate the two phases by centrifugation at 12 000 rpm for 2 min.

12 Draw off the lower chloroform phase and discard, add 10 μl 2 M NaOAc (pH 5.6) to the remaining supernatant.

13 Add 1 ml of cold (−20 °C) ethanol, mix well and leave in a dry ice/ethanol bath for 15 min.

14 Collect precipitate by centrifuging at 12 000 rpm for 10 min and discard supernatant. Add 1 ml of 70% ethanol and recentrifuge for 5 min.

15 Pour off supernatant, dry DNA under vacuum and redissolve in 50 μl H_2O.[c]

Fig. 1.11 *Use of Eco RI digestion to check putative pBin19CaMV-cat constructs in E coli*
A Fragment sizes expected from an *Eco* RI digest of the two possible single insert orientations and from the four possible tandem ligations of two inserts.
B Results of an *Eco* RI digest of several miniprep DNAs from a ligation experiment. Lanes **2–5** and **7–9** contain the same fragments as predicted for conformation 2, and lane **11** contains those predicted for conformation 1. Lane **6** contains an additional 7 kb fragment, and lane **10** contains none of the expected fragments. The constructs in the latter two lanes have probably suffered deletions and/or other rearrangements during ligation.

Notes

a 8 h growth should be sufficient.

b All the solutions except the alkaline SDS should be kept on ice.

c The DNA should be clean enough for restriction digests and 10 μl should be sufficient for each digest.

1.9 ANALYSIS OF MINIPREP DNA BY RESTRICTION DIGESTION AND AGAROSE GEL ELECTROPHORESIS

1.9.1 Background

Details on the use of restriction enzymes to digest DNA and the analysis of restriction fragments by electrophoresis on agarose gels are presented in 5.2 and 5.3 respectively and will not be repeated in the present section.

Most purified plasmid DNA samples require two to three times the enzyme activity needed to cleave an equivalent amount of purified test DNA (such as bacteriophage λ DNA) upon which restriction enzyme activity is usually assayed. Plasmid minipreps may contain contaminants which reduce the activity of these enzymes, and the concentration of plasmid DNA, together with any contaminating chromosomal DNA, is variable. Thus, miniprep DNA is usually digested in the presence of a 5–10-fold excess of enzyme for 1 h, or a 2–3-fold excess for several hours. Restriction digestion of plasmids often yields fragments which co-migrate with, and are thus obscured by, the large amounts of RNA that invariably contaminate minipreps. These may be visualized by treating completed digests with DNase-free RNase[b] before electrophoresis.[c]

When checking out putative recombinant plant transformation vector plasmids to determine whether the required insert has been obtained, restriction enzymes should be chosen which generate a unique and easily recognizable set of fragments from the desired vector/insert configuration. This generally requires extensive restriction maps of both vector and insert. Whenever possible, parent plasmids should be digested to yield fragments which correspond to some of those produced from the restriction of the recombinant plasmid, since co-migration of fragments is always more convincing than sizing by comparison with molecular-weight markers.[a]

In this section, an example analysis is presented of minipreps of putative recombinant plasmids representing the ligation of the 4.6 kb CaMV-*cat* *Hind* III fragment into the *Hind* III site of the plant transformation vector pBin19 (Fig. 1.11). As can be seen from Fig. 1.11A, an *Eco* RI digest of miniprep plasmid DNA yields information about both the number and orientation of inserts contained in the construct. The expected restriction fragment sizes for the desired construct are either 11.7, 2.25 and 0.65 kb, or 12.25, 1.70 and 0.65 kb. These can be seen in Fig. 1.11B, lanes 2–5, 7–9 and 10 respectively. The presence of extra bands, or the absence of expected bands, indicates that the recombinant plasmid has a

structure other than that which was desired and strains containing these plasmids may be discarded.

Notes

a Routinely λ DNA cut with *Hind* III or *Eco* RI can be used as size markers. The molecular weights of the restriction fragments obtained are given in 5.5.3 note c.

b To each digest add 2 μl of heat-treated RNase A (BCL) solution and incubate at room temperature for 2 min. Add 5 μl gel-loading buffer and store on ice prior to loading agarose gel for analysis.

c In general, approximately 0.1 μg DNA is loaded for each band that is expected, but as little as 40 ng may be seen on a gel.

1.10 CONJUGATION OF RECOMBINANT PLASMIDS INTO *AGROBACTERIUM*

1.10.1 Background

The conjugations that are required to transfer the plasmid of interest from *E. coli* to *Agrobacterium* are most conveniently carried out by a triparental mating (Van Haute *et al.*, 1983). Two conjugation systems are generally used: the first for intermediate vectors based on Col E1 replicons for use with the co-integrative-type vectors, and secondly, for RK2 replicons on which many binary vectors are based. Conjugation 'helper' plasmids specific for both types of replicon are used to provide mobilization (*mob*) and transfer (*tra*) functions in *trans*, either on separate replicons, or integrated into specific *Agrobacterium* host strains (Koncz and Schell, 1986). For the conjugation system to function, the cloning vectors must contain a specific origin of transfer (*oriT*) and an activation site (*bom*) on which the *tra* and *mob* gene products act.

Col E1 replicons

During the triparental mating, the helper plasmids (commonly pGJ28 and pR64*drd*11) are transferred at high frequency into the *E. coli* strain carrying the recombinant plasmid. The helper plasmids then effect the transfer of all three plasmids into *Agrobacterium* at frequencies of around 10^{-4}–10^{-5} (Fig. 1.5B). In a triparental mating (Van Haute *et al.*, 1983) all three strains are mixed together and the desired *Agrobacterium* transconjugant selected for. Alternatively, this procedure can be carried out by performing firstly a biparental mating between the *E. coli* strain containing the intermediate vector and the *E. coli* strain having the helper plasmids, before a second mating between the product of this first conjugation event and the *Agrobacterium* strain carrying the *vir* helper functions. In systems with *cis*-acting *vir* regions (e.g. pGV3850), vector sequences are maintained by homologous recombination into the resident virulence helper plasmid.[a]

Co-integration occurs at a rather low frequency and so much fewer stable transconjugants are normally obtained with *cis*-type than with binary vectors.

RK2 replicons

Most binary system cloning vectors have a wide host range RK2 origin of replication and are usually conjugated using helper functions supplied by pRK2013 (Ditta *et al.*, 1980). Mobilization is again achieved by either two biparental matings, or, more commonly, a single triparental mating. The mobilization frequency is normally in the range of 10^{-2}–10^{-3} and is therefore more efficient than that achieved with Col E1 replicons. Unlike intermediate vectors, the RK2 replicons are maintained in *Agrobacterium* (under selection) without having to rely on co-integrate formation and so transconjugants are selected at a much higher frequency.

Many commonly used acceptor strains of *Agrobacterium* contain a chromosomally located rifampicin (Rf)-resistance gene, and most examples of both types of cloning vectors (Tables 1.1 and 1.2) contain prokaryotic-type antibiotic-resistance markers other than Rf, including Km, Sm, Tc and Am. Thus, transconjugant agrobacteria containing recombinant sequences can be selected as Rf- and Km-/Sm-/Tc-/Cb-resistant colonies. In practice some rearrangement or deletion of transferred sequences may occur and thus it is a necessary precaution to check constructs in transconjugants (1.11 and 1.12). As an example the triparental matings aimed at inserting recombinant pBin19 transformation vectors into the *vir* helper strain LBA4404 will be considered. *Agrobacterium* transconjugants containing putative pBin19CaMV-*cat* can be selected for on NA + Rf + Km plates. Neither of the two *E. coli* strains are resistant to Rf and so the only colonies formed on these plates will be *Agrobacterium*. Kanamycin selects for the presence of pBinl9 sequences in transconjugants.

1.10.2 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).
- Sterile toothpicks.
- 37 °C and 28 °C environmental orbital shakers with clips for Universals.
- 28 °C static incubator.

Bacteria

- Fresh bacterial plates:
  - A Helper strain (e.g. HB101 : : pRK2013).
  - B *Agrobacterium* receptor strains (e.g. LBA4404).
  - C Colonies from transformation selection plates containing confirmed recombinant plant transformation vectors (e.g. pBin19CaMV-*cat*).

Solutions

- Liquid media (5 ml in Universal bottles):
  - A NB + selective antibiotics for *E. coli* helper and donor strains (e.g. 50 μg/ml Km for HB101 : : pRK2013 and 100 μg/ml Km for pBin19CaMV-*cat*).
  - B NB + selective antibiotics for *Agrobacterium* recipient strains (e.g. 100 μg/ml Rf for LBA4404).
  - C NB.
- Solid media (9 cm Petri dishes with 20 ml of medium):
  - A NA (for conjugation).
  - B NA transconjugant selection plates (e.g. NA + Rf + Km to select pBin19 transconjugants in LBA4404).

1.10.3 Procedure

1 Pick single colonies of donor (i.e. those which appeared to contain correct constructs from restriction analysis) and helper strains of *E. coli* into 5 ml of NB + selective antibiotics and shake overnight at 37 °C.

2 Pick single colonies of recipient strains of *A. tumefaciens* into 5 ml of NB + selective antibiotics and shake overnight at 28 °C.

3 Pipette 100 μl of each of the three strains to be mated (i.e. a donor, helper and recipient) onto one NA plate and mix by spreading. Repeat to give each triparental mating in duplicate and incubate overnight at 28 °C.

4 Using a sterile loop remove 2 'streaks' of cells from each plate, resuspend in 500 μl NB and streak out on 2 × NA[b] plates containing selective antibiotics both for *Agrobacterium* and the presence of the introduced recombinant sequences.

5 Incubate for 2 days at 28 °C, during which time single colonies of transconjugant *Agrobacterium* should appear.[c]

Notes

a *Agrobacterium* can also be selected from *E. coli* on the basis of its ability to grow on minimal medium, but this selection requires care as *E. coli* can grow on the small amounts of nutrients that are introduced by poor streaking technique.

b Since the introduction of sequences into *cis* vectors involves recombination as well as conjugation, approximately 1000-fold fewer transconjugant *Agrobacterium* colonies will be produced than would be expected from an equivalent binary system.

c *Agrobacterium* is incubated at 28 °C because Ti plasmids are not stably maintained at temperatures above 32 °C.

1.11 ISOLATION OF TOTAL NUCLEIC ACIDS FROM *A. TUMEFACIENS*

1.11.1 Background

After transconjugants have been selected the vector sequences have to be checked again in *Agrobacterium* to ensure that rearrangements or deletions have not occurred during conjugation. In addition, when using *cis*-type vectors such as pGV3850 it is necessary to demonstrate that the intermediate vector sequences have co-integrated into the plant transformation vector, to determine their orientation and to check that co-integration has not resulted in rearrangements of the inserted sequences. Non-oncogenic, *cis*-type Ti plasmid transformation vectors are low copy number megaplasmids of ≈180–250 kb. In order to characterize such large plasmids which require specialized techniques to isolate intact, it is necessary to carry out a total nucleic acid preparation followed by Southern analysis of this DNA to identify specific sequences. Likewise sequence characterization of binary vectors, also low copy number plasmids when maintained in *Agrobacterium*, requires preparation of total nucleic acids.

The extraction procedure uses only small volumes of overnight cultures and involves an enzymatic digestion of the agrobacteria followed by removal of proteins from the extract by treatment with phenol/chloroform and concentration of nucleic acids by ethanol precipitation. The technique outlined below has been used successfully with a range of *Agrobacterium* strains. Restriction enzyme digestion of *Agrobacterium* total DNA and an example Southern analysis of plasmid sequences contained in several *Agrobacterium* total nucleic acid minipreps are presented in the next section (1.12).

1.11.2 Materials

Equipment/consumables

- General molecular biology equipment (Appendix 1 [I]).
- Sterile toothpicks.

Bacteria

- Putative transconjugants from triparantal matings.
- Recipient strain of *Agrobacterium* as control (e.g. LBA4404).

Solutions

- NB + selective antibiotics (5 ml in Universal bottles) for growth of transconjugants and control strains (see 1.10.2 for examples).
- 5% 'Sarkosyl' (Sigma, *N*-lauroylsarcosine, sodium salt) in TE.
- 5 M NaCl (292.2 gm/l).
- TE: see 1.4.2.

- Pronase solution (5 mg/ml pronase (Sigma) in TE). This must be predigested at 37 °C for 2 h and stored at −20 °C.
- Phenol/chloroform (1 : 1); see 1.5.2.1.
- 100% and 70% ethanol (−20 °C).

1.11.3 Procedure (modified from Dhaese *et al.*, 1979)

1 Using a sterile toothpick, pick a single colony of *Agrobacterium* into 5 ml of NB containing selective antibiotics and shake overnight[a] at 28 °C.

2 Transfer 1.5 ml of cultures to microfuge tubes and collect cells by centrifugation at 12 000 rpm for 5 min.

3 Remove supernatant and resuspend cells in 300 µl TE.

4 Add 100 µl 5% Sarkosyl in TE and mix.

5 Add 150 µl 5 mg/ml pronase and mix.

6 Incubate at 37 °C for 1 h.

7 Add 500 µl phenol/chloroform and, using a Gilson P1000 set at 1.0 ml, gently pass mixture through a pipette tip 5 times in order to shear the viscous mass of membranes present in the bacterial lysate.

8 Separate the aqueous and organic phases by centrifugation at 12 000 rpm for 5 min.

9 Remove aqueous supernatant to a new tube and extract 3 times by adding 500 µl phenol/chloroform, gently emulsifying the phases, centrifuging at 12 000 rpm for 5 min and removing the upper aqueous phase to a new tube.

10 Estimate the volume of the aqueous phase and add 0.05 volumes of 5 M NaCl to give a final concentration of 0.25 M. Add 3 new volumes of cold (−20 °C) ethanol and leave at −20 °C for 1–2 h.

11 Collect the nucleic acid by centrifugation at 12 000 rpm for 10 min. Pour away supernatant, wash pellet with 1 ml of 70% ethanol and again collect nucleic acids by centrifugation (12 000 rpm, 10 min).

12 Carefully remove supernatant and partially dry pellet under vacuum.[b]

13 Resuspend pellet in 50 µl H_2O.

Notes

a Since typical generation times of *Agrobacterium* are about 2 h and those of *E coli* are about 20 min, both liquid cultures and plates containing *Agrobacterium* may have to be incubated for 2 days before the required cell growth is achieved.

b Do not dry the nucleic acid pellet entirely as this will make it almost impossible to resuspend in water.

1.12 RESTRICTION ENZYME DIGESTION OF *AGROBACTERIUM* TOTAL DNA

1.12.1 Background

Details on the digestion of DNA by restriction enzymes and Southern blot analysis of DNA sequences are presented in Chapter 5. However, some particular points specifically associated with the analysis of vector sequences in agrobacteria are presented in the present section.

Restriction digestion of total DNA from *Agrobacterium* coupled with Southern blot analysis can be used not only to confirm that a specific foreign DNA sequence is co-integrated into a *cis*-type Ti plasmid-derived vector, or maintained in a *trans*-type binary vector, but also to determine its organization. Restriction enzymes should be chosen that produce a characteristic restriction fragment pattern. In the case of co-integrative vectors it is important to chose restriction enzymes and hybridization probes which allow the visualization of both internal sequences and junction fragments. As with any type of Southern blot analysis the gel should also include some control DNA samples, for example the original vector molecules as negative controls and purified plasmid from which the insert DNA was obtained as positive controls, as well as radiolabelled molecular weight markers (see Chapter 5).

As with the *E coli* minipreps, the total *Agrobacterium* DNA is not very pure and is not at a known concentration. In general, 10 μl is sufficient for restriction digestion and Southern blotting, and the following points should be noted:

- Ensure that the nucleic acid is completely redissolved. This avoids smearing on the agarose gel and difficulties with cutting with restriction enzymes. A successful digest should result in a smear of DNA containing visible bands following electrophoresis (Fig. 1.12A, lanes 3–8).
- Incomplete digests are a common problem and may be overcome by:
  - A Increasing the final volume of the digest.
  - B Increasing the amount of the restriction enzyme used.
  - C Increasing the time of digestion (digests can be left overnight if this is convenient).
  - D Carrying out an additional phenol/chloroform wash of the preparation, precipitating the nucleic acid with ethanol and washing with 70% ethanol prior to digestion with enzymes.

A typical analysis based on our example is shown in Fig. 1.12A. Lanes 3–7 (Fig. 1.12A) contain *Hind* III-digested DNA from transconjugants of five of the *E. coli* transformants shown in Fig. 1.11 which were found to harbour the correct constructs. Lane 2 contains the *Hind* III CaMV-*cat* fragment as a positive hybridization control and lane 8 contains *Hind* III-digested LBA4404 DNA as a negative control. Note that in all lanes the DNA is evenly digested, with many discrete bands visible, and has little high molecular weight DNA remaining at the well end of the gel. Poorly digested DNA will tend to streak, produce little, if any, discrete bands and will be composed of mainly high molecular weight species.

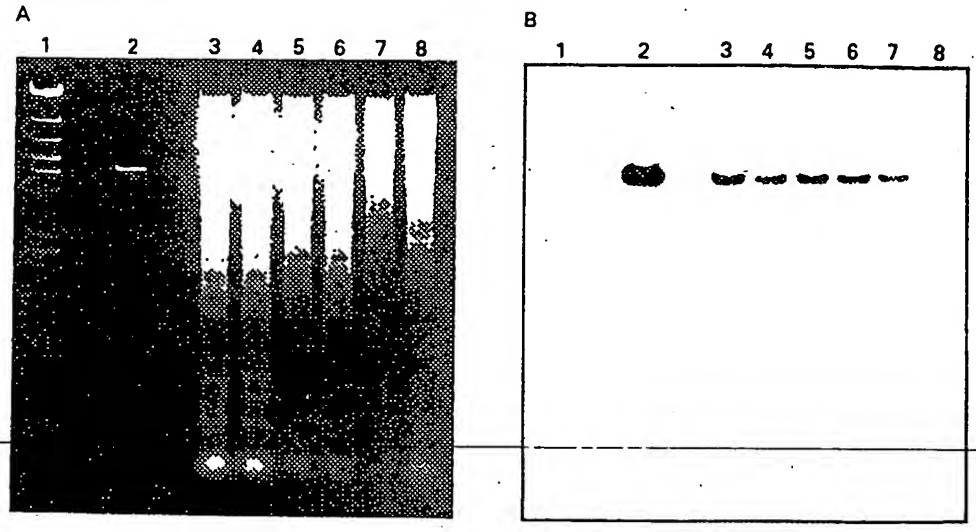

Fig. 1.12 *Analysis of pBin19CaMV-cat in LBA4404 by Southern blotting*
A Ethidium bromide stained agarose gel. Lane **(1)** molecular weight markers composed of λ DNA cut with *Hind* III and *Hind* III + *Eco* RI, **(2)** 4.6 kb *Hind* III CaMV-*cat* fragment; **(3–7)** *Hind* III-digested DNAs from different transconjugants, **(8)** *Hind* III-digested LBA4404 DNA
B Southern blot of the gel shown in A hybridized to the *cat* probe described in 3.2.3.2.

The gel in Fig. 1.12A was Southern blotted and hybridized to a *cat* probe, as described in Chapter 5. The resultant autoradiograph is shown in Fig. 1.12B. Strong and specific hybridization can be seen in both the positive control and lanes 3–7, whereas none is observed in LBA4404 DNA (lane 8). The *Hind* III digestion has liberated a fragment which co-migrates with and has homology to the *Hind* III CaMV-*cat* fragment. Therefore it can be concluded that all the transconjugants examined contain the desired constructs which appear to have maintained their integrity throughout the conjugation process.

APPENDIX 1 [I]

General molecular biology equipment

- Gilson Pipetteman; P20, P200, P1000 (Anachem Ltd).
- Gilson tips in racks (autoclaved and dried):
  Yellow (P20, P200)
  Blue (P1000).
- Microcentaur centrifuge (MSE Scientific Instruments Ltd.).
- Eppendorf microcentrifuge tubes (1.75 ml) sterilized in a foil-covered 500 ml Pyrex beaker (W. Sarstedt & Co.).
- Ice bucket (and source of crushed ice).
- Dry ice/EtOH bath (and source of dry ice).
- Polystyrene tile float for Eppendorf tubes.
- Static water baths (Gallenkamp).
- Boiling water bath (Gallenkamp).
- Freezers; −20 °C, −80 °C.
- Fridge.
- Electronic balance and weighing boats.
- Magnetic stirrer and stirring bars.
- Whirlimixer.
- Spatula, forceps, scalpels, scissors etc.
- Fine marker pens.
- Bacterial loop.
- Bunsen burner.
- Bacterial spreader (glass).
- Large beaker with bacteriocide for contaminated solutions.
- Timer.
- 'Cinbins' (Metal Box PLC):
  A Bacterially-contaminated tips.
  B ^{32}P-contaminated tips.
  C ^{35}S-contaminated tips.
  D ^{14}C-contaminated tips.
- Kleenex tissues (Kimberley–Clark Ltd.).
- Absorbant paper towels (Kimberly–Clark Ltd.).
- Benchkote (Whatman).
- Nescofilm (BDH).
- Saran wrap (Dow Chemical Company Ltd.).
- Electrical, masking and radiation hazard tape.
- Scotch Magic tape (3M United Kingdom PLC. (UK)).
- Disposable rubber gloves (e.g. Surgikos Ltd; Microtouch.).
- Plastic disposable Petri dishes (Sterilin Ltd.).
- Plastic disposable Universal bottles (Sterilin Ltd.).
- Glass Universal bottles.
- Conical flasks; 2 litres, 500 ml and 250 ml.

- Plastic cuvettes (3 ml, 1 ml and 0.5 ml).
- Pyrex beakers and measuring cylinders (from 50 ml to 2 litres).

500 ml

APPENDIX 1 [II]

A Bacterial strains used in examples

Name	Description	Resistances	Reference
JM83	*E coli*	Sm	Messing and Vieira (1982)
C58Cl (pGV3850)	*A. tumefaciens*	Cb, Rf	Zambryski *et al.* (1983)
GJ23	*E. coli*	Km, Tc	Van Haute *et al.* (1983)
LBA4404	*A tumefaciens*	Rf	Hoekma *et al.* (1983)
HB101::pRK2013	*E. coli*	Km	Ditta *et al* (1980)
MC1022::Bin19	*E coli*	Km	Bevan (1984)

B Antibiotics used for bacterial selection

Name	Selective concentrations for		Stock (mg/ml)	Solvent
	E coli (μg/ml)	*A. tumefaciens* (μg/ml)		
Ampicillin (Ap)	50.0	—	25.0	H_2O
Carbenicillin (Cb)	100.0	100.0	50.0	H_2O
Kanamycin (Km)	100.0	100.0	100.0	H_2O
Rifampicin (Rf)	100.0	100.0	50.0	Methanol
Tetracycline (Tc)	15.0	2.5	12.5	Ethanol
Streptomycin (Sm)	12.5	300.0	900.0	H_2O
Spectinomycin (Sp)	50.0	100.0	50.0	H_2O
Cefotaxime (Cx)	—	250.0	250.0	H_2O

All antibiotics which are water soluble are sterilized by filtration (0.22 μm pore size) and then stored as frozen stock solutions at −20 °C. Kanamycin and carbenicillin are relatively stable and can often be stored for up to one month at 4 °C without degradation. Rifampicin and tetracycline should be weighed out into a sterile Universal and then an appropriate amount of methanol or ethanol added respectively. After vigorous shaking, the antibiotic should dissolve and will also be sterile. It is a good idea to only make up the amount of antibiotic solution that you intend to use over a 2–3 week period. It should also be noted that at room, or higher temperatures, and in the light, many antibiotics will quickly break down and become inactive.

Antibiotics may be added directly to liquid medium or to molten and cooled (45–55 °C) agar-solidified media as indicated in 1.3.5.

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